ÉTUDE DE LA FONCTION ANTIVIRALE DE PROTÉINES ARGONAUTES

par

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Chez les plantes, le mécanisme de l'ARN interférence régule l'expression des gènes par l'action des protéines Dicer-like (DCL) et Argonaute (AGO). De plus, l'ARN interférence fonctionne aussi comme un mécanisme de défense antiviral en ciblant l'ARN double brin dérivé du virus. La plante modèle, *Arabidopsis thaliana*, code pour 10 protéines AGO avec des fonctions spécialisées. Une fonction antivirale a été identifiée pour certaines d'entre elles contre différents virus. Néanmoins, mise en part AGO2, l'implication des autres AGOs dans la défense antivirale contre les Potexvirus n'est pas totalement élucider.

Au cours de mes travaux, j'ai démontré, à l'aide d'essais fonctionnels, que toutes les protéines AGOs possèdent la capacité intrinsèque de reconnaître et cibler l'ARN viral lorsque cet ARN n'est pas protégé à l'intérieur du complexe de réplication viral. Des essais génétiques ont permis de démontrer que la protéine AGO5 d'*Arabidopsis* contribue, conjointement avec AGO2, à l'immunité antivirale de cette plante contre PVX sauvage capable de former des complexes de réplication. De façon encore plus importante, nous démontrons que toutes les protéines AGO d'*Arabidopsis* possèdent la capacité intrinsèque de reconnaître et cibler l'ARN viral lorsque cet ARN n'est ni protégé par l'action d'un VSR ou par un complexe de réplication viral intact suggérant que la formation de ces complexes permet de protéger les ARN viraux de la machinerie antivirale de l'hôte.

De façon intéressante, nous démontrons que bien qu'AGO2 et AGO5 possèdent une activité antivirale contre PlAMV, un potexvirus, la répression de ce dernier chez *Arabidopsis* dépend majoritairement de la protéine AGO4.

L'étude de la variabilité de la séquence codante d'AGO2 chez différentes accessions d'*Arabidopsis thaliana* m'a permis d'identifier deux régions, dans la protéine AGO2, importantes pour l'activité antivirale de cette protéine et qui ont été soumises à la sélection

naturelle. La variabilité génétique retrouvée dans ces deux régions joue un rôle dans la détermination de la gamme d'hôte pour les virus végétaux. Ce genre de rôle n'a jamais été décrit pour le mécanisme de l'ARN interférence.

Mots-clés : Plante, ARN interférence, défense antivirale, Argonaute, complexe de réplication viral

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LISTE DES ABRÉVIATIONS

At	*Arabidopsis thaliana*
AGO	Argonaute
ADN	Acide désoxyribonucléique
AJ	Acide jasmonique
ARN	Acide ribonucléique
ARNdb	ARN double brin
At	*Arabidopsis thaliana*
BCTV	Beet curly top virus
BMV	Brome mosaic virus
CaMV	Cauliflower mosaic virus
CDS	Coding DNA sequence
CMV	Cucumber mosaic virus
CP	Coat protein
DCL	Dicer-like
DNA	Deoxyribonucleic acid
dpi	days post-infiltration / days post-infection
dsRNA	double stranded RNA
eGFP	enhanced GFP
ET	Éthylène
ETI	Effector triggered immunity
EV	Empty vector
F/R	Firefly/Renilla
GFP	Green fluorescence protein
H3	Histone 3

HA	Hemagglutinin
hcRNA	Heterochromatic siRNA
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IB	Immunobuvardage
IgG	Immunoglobuline G
IP	Immunoprécipitation
JA	Acide jasmonique
L	Local leaves
LUC	Luciférase
$MgCl_2$	Chlorure de magnésium
miRNA	microRNA
Nb	*Nicotiana benthamiana*
Nt	*Nicotiana tabacum*
NB-LRR	*nucleotide-binding, leucine-rich repeats*
NLS	signal de localisation nucléaire
nt	Nucleotide
NRPD	Nuclear DNA-dependent RNA polymerase
OD	Densité optique
p	Promoteur
P-bodies	*Processing bodies*
PAGE	*Polyacrylamide gel electrophoresis*
PAMP	*Pathogen-associated molecular pattern*
PCR	Polymerase chain reaction
PEPC	*Phosphoenol pyruvate carboxylase*
Pol	Polymréase
PLAMV	Plantago asiatica mosaic virus
PSTVd	Potato spindle tuber viroid
PTGS	Post-transcriptional gene silencing
PTI	PAMP-triggered immunity / Immunité induite par les PAMPs

PVX	Potato virus X
qRT-PCR	quantitative RT-PCR
RdDM	*RNA-Directed DNA Methylation*
RDR	*RNA-dependent RNA polymerase*
RISC	*RNA-induced silencing complex*
RNA	*Ribonucleic acid*
RT-PCR	Reverse transcription polymerase chain reaction
S	systemic leaves
S1	Streptavidin aptamer
SA	acide salicylique
SDS	sodium dodecyl sulfate
SGS3	*Suppressor of gene silencing 3*
sRNA	*small RNA*
sgRNA	*subgenomic RNA*
siRNA	*Small interfering RNA*
ssRNA	*single stranded RNA*
TBSV	Tomato bushy stunt virus
TCV	Turnip crinkle virus
T-DNA	*Transfer DNA*
TGB	*Triple gene block*
TGS	*Transcriptional gene silencing*
ToRSV	Tomato ringspot virus
TRV	Tobacco rattle virus
TuMV	Turnip mosaic virus
UV	ultraviolet
vARN :	ARN viral
VRC:	Viral replication complex
vsiARN:	Viral small interfering RNA
VSR	Viral suppressor of RNA silencing
WT	Wild type

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INTRODUCTION GÉNÉRALE

Les plantes, comme les animaux, sont constamment exposées aux attaques par d'autres organismes vivants dans leur environnement tel que les insectes, les nématodes, les protozoaires, les champignons, les bactéries, les virus et les viroïdes (Dreher et Callis, 2007). En raison de leur grande diversité, les agents phytopathogènes figurent parmi les menaces les plus graves

pour la sécurité alimentaire mondiale (Dangl et al., 2013). En effet, jusqu'à 30% des cultures sont perdues avant ou après la récolte, entre autres, dû aux maladies causées par des agents pathogènes (Jones et al., 2016).

Parmi les agents phytopathogènes, on estime que les phytovirus sont responsables de la moitié des maladies émergentes signalées chez les plantes cultivées (Anderson et al., 2004). Le contrôle des phytovirus est souvent dépendant de l'utilisation de pesticides de synthèse (Bragard et al., 2013). Cependant, une telle stratégie a de nombreux effets néfastes sur les écosystèmes ainsi que la santé humaine (Bragard et al., 2013).

Une des stratégies alternatives à l'utilisation de pesticides et autres produits chimiques est l'utilisation de variétés améliorées résistantes aux agents pathogènes. Pour ce faire, il est primordial de caractériser le système immunitaire végétal et le dialogue entre la plante et les agents pathogènes.

1.1 Le système immunitaire constitutif et induit

Dans la nature, le développement d'une maladie chez les plantes représente l'exception. En effet, bien que les plantes soient dépourvues d'un système immunitaire circulant et de cellules immunitaires spécialisées comme ceux retrouvés chez les mammifères, les plantes possèdent un système immunitaire complexe et efficace permettant, dans la plupart des cas, à contrer l'infection par les agents pathogènes (Spoel et Dong, 2012; Couto et Zipfel, 2016). Un grand avantage du système immunitaire végétale est la capacité de chaque cellule de détecter la présence d'agents pathogènes et ainsi de monter une réponse de défense de type cellule autonome. De plus, la résistance initiée localement au lieu même de l'attaque par l'agent pathogène peut, dans certains cas, se propager dans la plante entière dans les tissus systémiques et perdurer dans le temps afin de protéger contre une nouvelle attaque (Spoel et Dong, 2012).

L'immunité végétale est généralement classifiée en deux catégories; soit l'immunité constitutive et l'immunité induite. L'immunité constitutive, ou passive, en est une qui est non-spécifique et

qui est préformée avant même l'arrivée de l'agent pathogène. Elle a pour but de limiter l'entrée des agents pathogènes et inclut l'ensemble des barrières physiques (structurales) de la cellule végétale ainsi que des barrières chimiques (métabolites préformés) (Mauch-Mani et al., 2017). Par exemple, la cuticule, l'épiderme et la paroi secondaire sont des barrières physiques alors que les phytoalexines, les glucosinolates et les saponines sont des exemples de métabolites secondaires ayant des propriétés antimicrobiennes. Cette première ligne de défense constitue souvent un obstacle suffisant contre la plupart des agents pathogènes (Mauch-Mani et al., 2017). Cependant, certains agents pathogènes arrivent à franchir ce premier obstacle en produisant des enzymes lytiques pour dégrader cette barrière structurelle et accéder à la cellule (Boller and Felix, 2009).

Lorsque cette première barrière physique est altérée ou si un agent pathogène possède les outils nécessaires pour la surmonter, l'agent pathogène rencontrera une défense plus spécifique soit, la défense induite. Celle-ci est subdivisée en deux couches soit : une couche extracellulaire assurée par des récepteurs membranaires reconnaissant des éliciteurs généraux et une couche intracellulaire reconnaissant des effecteurs produits ou injectés par l'agent pathogène dans le cytoplasme de la cellule hôte (Jones et Dangl, 2006).

1.1.1 La résistance basale induite par les motifs moléculaires associés aux agents pathogènes ou PTI

Cette première couche de la défense induite repose sur la détection, dans l'apoplaste, d'éliciteurs généraux tel que les DAMPs (Damage-associated molecular pattern) ou les PAMPs (Pathogen-associated molecular patterns) (Musidlak et al., 2017). Les DAMPs sont certaines composantes dérivées de la paroi cellulaire végétale qui sont libérées suite à l'attaque par des agents pathogènes. Les PAMPs sont, quant à eux, des molécules hautement conservées retrouvées à la

surface des agents pathogènes tel que la flagelline, les lipopolysaccharides, le facteur d'élongation Ef-Tu, les peptidoglycans, la chitine, etc. Pour les phytovirus, aucun PAMP n'a été identifié à ce jour (Musidlak et al. 2017). Cependant, il a démontré récemment que certaines protéines virales, bien que peu conservées, peuvent enclencher la PTI (Zorzatto et al., 2015). De plus, il a été démontré que l'ARN bactérien et viral ainsi que l'ARNdb induisent aussi la PTI (Lee et al., 2016; Niehl et al., 2016)(Figure 1.1).

La reconnaissance de ces éliciteurs est assurée par des récepteurs situés à la surface de la cellule hôte, les PRRs (Pattern recognition receptors). Selon leur nature, ces récepteurs sont classifiés en deux familles soit, les RLKs (Receptor like kinase) et les RLPs (Receptor like protein) (Couto et Zipfel, 2016). Structurellement, les PRRs sont assez conservés. Les RLKs sont des protéines composées d'un ectodomaine liant potentiellement le ligand, d'un domaine transmembranaire et d'un domaine kinase intracellulaire. Les RLPs possèdent une même structure de base sauf pour le domaine intracellulaire qui ne possède ni activité kinase ni activité de signalisation connue (Couto et Zipfel, 2016). Pour cette raison, on pense que les RLP doivent nécessairement s'associer avec d'autres récepteurs de type kinase pour la transduction du signal (Gust et Felix, 2014) (Figure 1.1). Les PRRs occupent un rôle crucial pour le succès de PTI puisque leur délétion rend les plantes plus vulnérables à différents microorganismes (Huang et Zimmerli, 2014).

La reconnaissance des MAMPs et des DAMPs par les PRRs induit une cascade de signalisation qui ultimement enclenche le système de défense basal des plantes. Dans les étapes précoces de la PTI, on observe une augmentation de la concentration cytosolique en ions Ca^{2+}, une production d'espèces réactives de l'oxygène (ROS) et l'activation des voies des MAPKs (Mitogen-activated protein kinase) ou CDPK (Calcium-dependent protein kinase) (Meng et Zhang, 2013). En aval de cette réponse, il y aura une reprogrammation transcriptionnelle induisant la transcription de gènes impliqués dans la défense (tel que les protéine PR : Pathogenesis related), de la production de métabolites secondaires antimicrobiens (tel que les phytoalexines), de la déposition de callose ainsi qu'une production accrue d'hormones (tel que

l'éthylène (ET), l'acide jasmonique (JA) et l'acide salicylique (SA)) (Boller et Felix, 2009; Huang et Zimmerli, 2014; Lee et al., 2015; Bigeard et al., 2015) (Figure 1.1).

Dans la majorité des cas, la PTI est souvent suffisante pour contrer l'invasion par les microorganismes non adaptés à l'hôte conduisant donc à la résistance face à ce microorganisme. Pour échapper à la première couche du système de défense des plantes, un bon nombre d'agents pathogènes se sont, à leur tour, adaptés à l'hôte et ont développé des facteurs de virulence qui sont délivrés à l'intérieur de la cellule hôte et qui altèrent la structure de la cellule et ses fonctions (Cui et al., 2014). Ces protéines contribuent à la virulence d'un agent pathogène. Le mécanisme principal des effecteurs est d'interférer avec la PTI permettant ainsi la multiplication de l'agent pathogène et la mise en place de la maladie (Cui et al., 2014). Cette contre-attaque par l'agent pathogène est couramment appelée : susceptibilité induite par un effecteur (ETS).

Les effecteurs sont sécrétés dans l'espace apoplastique ou dans le cytoplasme en utilisant différents mécanismes d'injection selon le groupe d'agents pathogènes. Par exemple, certaines bactéries, tel que *Pseudomonas syringae*, délivrent leurs effecteurs dans le cytoplasme de la cellule hôte via un système de sécrétion de type 3 (T3SS); alors que les effecteurs des oomycètes sont injectés grâce à présence d'un peptide signal et un motif RxLR (Dou and Zhou, 2012). Les virus, quant à eux, ne possèdent pas de facteurs de virulence classiques. Cependant, les protéines virales, tel que les protéines de capside et des polymérases, peuvent tout de même être reconnue par la plante (Zvereva et Poggin, 2012).

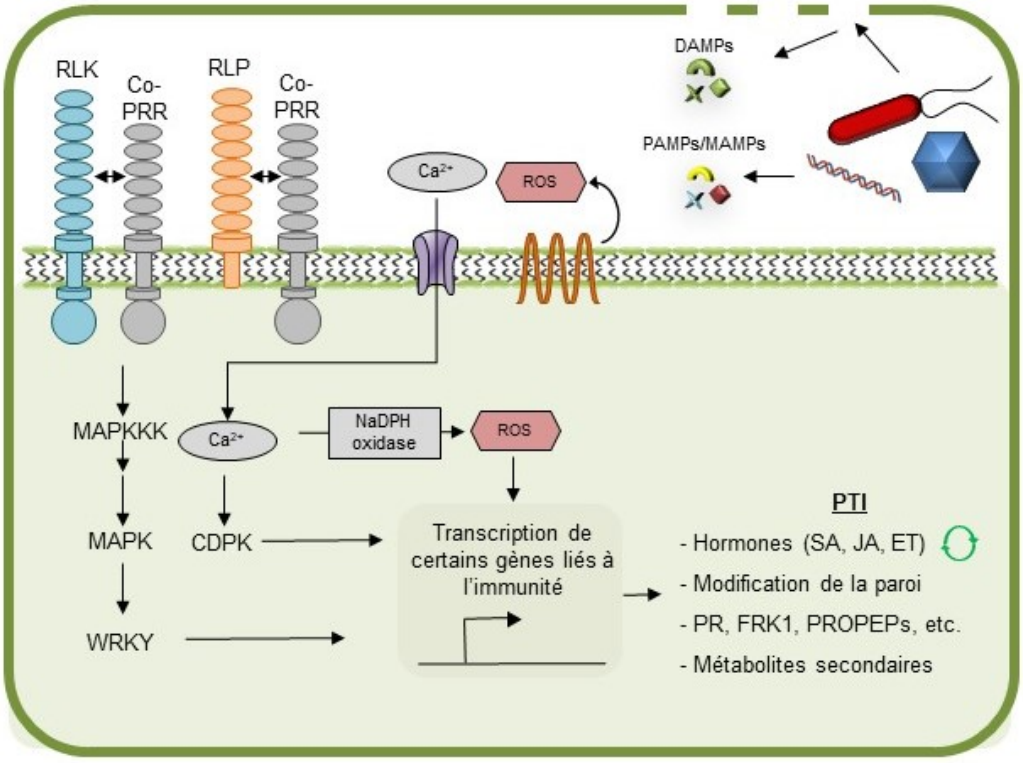


Figure 1.1 Schématisation de la PTI, l'immunité basale induite par les PAMPs/DAMPs. La perception des PAMPs ou des DAMPs par les PRRs, des récepteurs membranaires, induit la mise en place de la réponse de défense basale chez les plante, la PTI. Cette perception provoque une cascade de signalisation complexe menant à la reprogrammation transcriptionnelle de la cellule afin d'induire l'expression de gènes liés à la défense, la production de composés anti-microbiens, une modification de la composition de la paroi et une modification des concentrations de certaines hormones tel que l'acide salicylique (SA), l'acide jasmonique (JA) et l'éthylène (ET).

1.1.2 La résistance induite par les effecteurs ou ETI

Dans une course à l'armement, les plantes ont élaboré des récepteurs intracellulaires, protéines de résistance, permettant une détection spécifique de la présence d'un effecteur ou d'une perturbation d'une protéine ciblée par les effecteurs. Ces récepteurs immunitaires sont des protéines de type NB-LRR qui sont codées par des gènes de résistance (gène R). Selon la nature

de leur domaine N-terminal, ces protéines sont divisées en deux classes distinctes soit, les CC-NB-LRR (CNL) et les TIR-NB-LRR (TNL). Bien qu'il s'agisse d'une des familles de gènes les plus variables chez les végétaux, les NB-LRR sont structurellement assez conservés. Ces protéines sont constituées d'un domaine LRR (Leucine rich repeat) en C-terminal, un domaine NB-ARC central et d'un domaine variable en N-terminal se limitant soit à un domaine TIR (Toll-Interleukine1 receptor), soit à un domaine CC (Coiled coil). En plus de servir de plateforme de détection d'un effecteur ou d'une protéine de l'hôte modifiée par l'effecteur, ces domaines en N-terminal sont impliqués dans la signalisation de la réponse de défense (Collier and Moffett, 2009; Cui et al., 2015).

Au début de la réponse médiée par les NB-LRRs, on observe une accumulation d'ions Ca^{2+} et de ROS qui mène à l'activation des voies des CDPK et MAPKs. Finalement, on observe une reprogrammation transcriptionnelle qui chevauche celle de la PTI (Tsuda and Katagiri, 2010; Spoel and Dong, 2012; Cui et al., 2015)(Figure 1.2 (A)).

Cependant, l'intensité et la durée d'activation de ces mécanismes seront plus forts lors de l'ETI comparativement à la PTI (Tsuda and Katagiri, 2010; Spoel and Dong, 2012) (Figure 1.2 (B)). La voie de l'ETI diffère aussi de la PTI sur sa dépendance sur les intermédiaires de signalisation tel que le SA, et les protéines NPR1, EDS1 et NDR1 (Cui et al., 2014). De plus, l'intensité et la persistance de l'ETI culmine souvent vers une réaction hypersensible (HR), un type de mort cellulaire programmée localisée permettant de restreindre la dispersion de l'agent pathogène (Jones and Dangl, 2006).

Outre ces réponses locales, la PTI et ETI peuvent déclencher la résistance systémique acquise (SAR). Il s'agit d'une défense dans les tissus non-infectés de la plante qui est similaire à la défense locale et qui inclut les voies hormonales, l'induction de gènes liés à la défense et une résistance à large spectre et durable face à l'invasion subséquente par un agent pathogène (Spoel and Dong, 2012; Kachroo and Robin, 2013). Cette mémoire immunitaire peut même être transmise à la progéniture et perdurer pendant quelques générations.

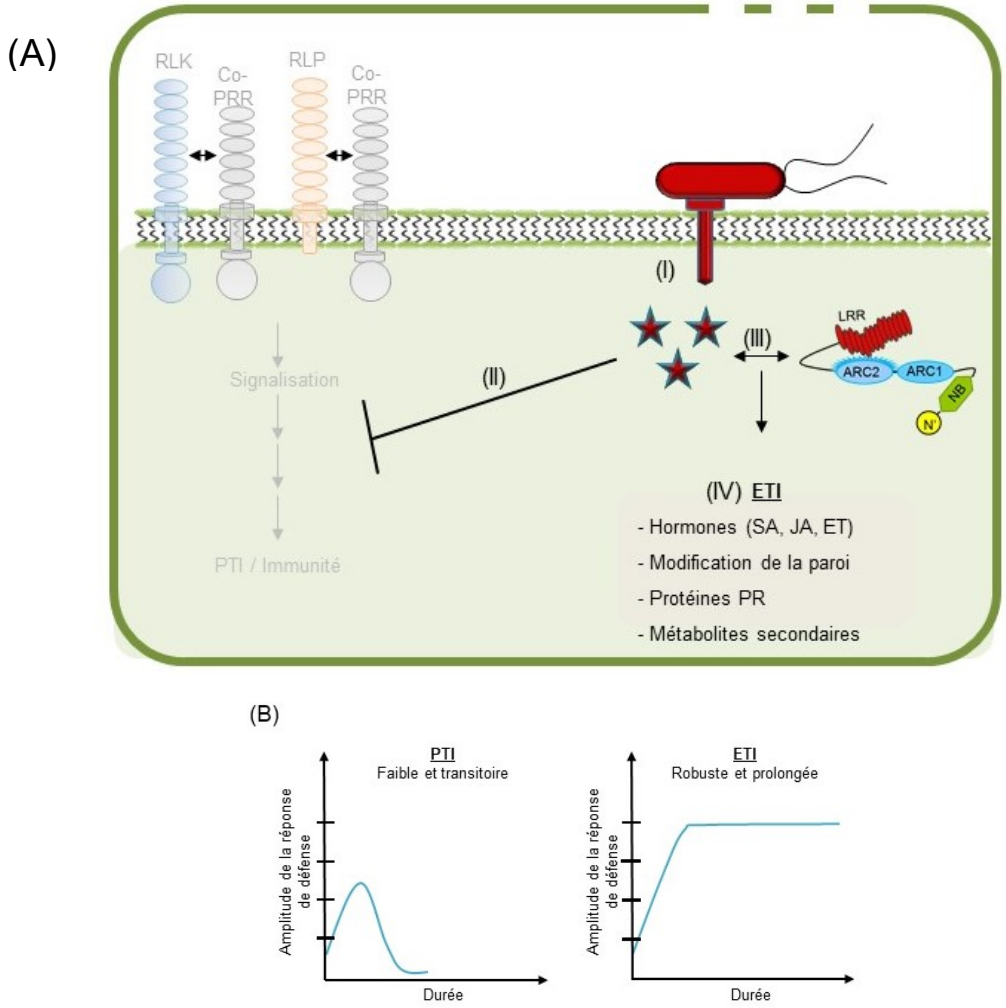


Figure 1.2 Schématisation de l'ETI, l'immunité induite par les effecteurs ou ETI.

(A) (I) Certains agents pathogènes mieux adaptés possèdent la capacité d'injecter dans le cytoplasme de la cellule hôte des facteurs de virulence, par exemple via un T3SS. (II) Ces protéines peuvent contourner, atténuer ou compromettre la réponse basale, la PTI. Ceci permet à l'agent pathogène de coloniser son hôte et d'induire la maladie. (III) Les plantes possèdent des récepteurs intracellulaires, des protéines NB-LRRs, permettant la détection de l'effecteur ou de l'effet de celui-ci sur une protéine de l'hôte. L'ETI culmine à une réponse de défense très similaire à celle de la PTI. (B) L'intensité et la durée d'activation de ces mécanismes seront plus forts lors de l'ETI.

1.2 La résistance récessive contre les virus

Bien que les virus codent pour un certain nombre de protéines essentielles (par exemple, la protéine de réplication, de la capside, les protéines de mouvement), la capacité d'encodage de leur génome est limitée. Ils dépendent donc de nombreux facteurs de l'hôte pour établir une infection (Nagy and Pogany, 2012; Wang, 2015). Dès 1986, il a été proposé par Ronald Fraser que la résistance contre les virus pourrait résulter de mutations ou d'une perte des composantes de l'hôte requises pour la réplication virale (Fraser, 1986). Depuis, plusieurs études ont démontré que la résistance récessive est souvent associée à la perturbation ou l'absence de facteurs de l'hôte nécessaires à l'achèvement du cycle de réplication du virus (Galvez et al., 2014). Parmi les gènes conférant la résistance chez les plante, les gènes récessifs jouent un rôle important dans la défense des plantes contre les virus et constituent environ la moitié des gènes antiviraux connus (Sanfaçon, 2015).

Une des premières étapes du cycle de réplication viral est la traduction des ARN viraux (vARN). Les virus n'encodent normalement pas les facteurs de traduction canoniques, mais ont développé plusieurs stratégies pour détourner les facteurs de traduction de leurs hôtes et favoriser la traduction de l'ARN viral au détriment des ARNm endogènes (Simon and Miller, 2013; Zhang et al., 2015; Sanfaçon, 2015). La majorité des gènes récessifs impliqués dans les interactions plante-virus codent pour des facteurs d'initiation de la traduction eucaryote (eIFs) de la famille 4E ou 4G, principalement eIF4E, eIF4G et leurs isoforms (Kang et al., 2005; Sanfaçon, 2015; Hashimoto et al., 2016; Machado et al., 2017). L'implication de eIF4E et eIF4G dans la résistance des plantes face aux virus a été reportée chez plusieurs espèces et contre de nombreux genre de virus, tel que les potyvirus, les tombusvirus, carovirus, sobemovirus, waikavirus, carmovirus, cucumovirus (Machado et al., 2017).

Plus récemment, des études ont démontré que ce type de résistance pourrait aussi être impliqué dans la défense contre les potexvirus (Hashimoto et al., 2016; Keima et al., 2017). En effet, il a été montré que des mutations ou la délétion de deux isoformes de eIF4E, EXA1 et CBP, causent

une diminution de l'accumulation de PlAMV et limite son mouvement de cellule-en-cellule (Hashimoto et al., 2016; Keima et al., 2017).

1.3 La résistance contre les virus médiée par les lectines – une résistance dominante

Les lectines végétales sont des protéines qui se lient de manière réversible aux hydrates de carbone et jouent un rôle important dans le développement et la résistance des plantes. Grâce à la liaison des ligands glucidiques, les lectines sont impliquées dans la perception des signaux environnementaux et leur traduction dans les réponses phénotypiques (Esch et Schaffrath, 2017). Évidemment, de par leur capacité à lier des ligands glucidiques, il n'est pas surprenant de voir que ces protéines sont impliquées dans l'immunité contre les bactéries, les oomycètes et les insectes (Lannoo et Van Damme, 2014). Plus récemment, des protéines *jacalin-like* ont été identifiées comme des facteurs importants dans la défense antivirale chez les plantes (Chisholm et al., 2000; Yamaji et al., 2012). La présence de RTM1 chez Arabidopsis inhibe le mouvement systémique d'un potyvirus, le Tobacco etch virus (TEV) (Chisholm et al., 2000). Le gène *JAX1* confère, quant à lui, la résistance contre les Potexvirus chez *Arabidopsis* (Yamaji et al., 2012).

1.4 L'ARN interférence

L'ARN interférence réfère collectivement à divers processus basés sur l'ARN résultant en l'inhibition, de façon séquence-spécifique, de l'expression génique soit au niveau transcriptionnel (TGS : *Transcriptional gene silencing*) ou au niveau post-transcriptionnel (PTGS : *Post-transcriptional gene silencing*), en déstabilisant l'ARN ou en inhibant sa traduction (Brodersen et Voinnet, 2006). Ce mécanisme possède de nombreux rôles, y compris la régulation du développement, la réponse au stress, ou la défense contre les acides nucléiques envahissants tels que les transposons, les virus et les viroïdes (Pumplin et Voinnet, 2013).

Tel qu'on le comprend actuellement, le mécanisme de l'ARN interférence est déclenché par la présence de structure d'ARN double brin (ARNdb) dans la cellule (Figure 1.3, (I)). Ces ARNdb sont coupés en plus petits segments par des endoribonucléases, les Dicer-like, pour produire des

duplexes courts d'ARN (sARNs), tels que des microARN (miARN) ou de courts ARN interférents (siRNA) d'une longueur de 21-24 nucléotides (nt) arborant des extrémités 3'-hydroxyle et 5'-phosphate, et 2-nt saillants à l'extrémité du duplexe (Bernstein et al., 2001; Hamilton and Baulcombe, 1999; Khvorova et al., 2003; Schwarz et al., 2003)(Figure 1.3, (II)). Pour cette action, certaines DCLs requièrent la collaboration des *Double-stranded RNA binding proteins* (DRBs), protéines liant l'ARNdb (Hiraguri et al., 2005; Eamens et al., 2012a, b). Ces sARNs sont ensuite méthylés à leur extrémité 3' par la protéine HUA Enhancer 1 (HEN1). Cette méthylation protège les sARNs contre l'uridylation et la dégradation de ces derniers. (Park et al., 2002; Yang et al., 2006) (Figure 1.3, (II)). Par la suite, le duplexe ($sARN_{passager}$-$sARN_{guide}$) est incorporé dans le complexe RISC (*RNA-induced silencing complex*) composé minimalement d'une protéine Argonaute (AGO). Les différences thermodynamiques dans les stabilités d'appariement de bases des extrémités 5' des deux brins d'ARNsi du duplexe déterminent quel brin de siRNA est assemblé dans le RISC. Le brin ayant une extrémité 5' moins stable thermodynamiquement sera conservé dans le complexe, c'est le brin guide. L'autre brin, le brin passager, sera clivé et supprimé (Figure 1.3, (III)). Le complexe RISC ainsi programmé cible ensuite les acides nucléiques complémentaires au sARN incorporé. L'interaction d'appariement de bases qui se produit entre les sARN et les cibles est responsable de la spécificité de séquence élevée de l'ARN interférence. Suite à la reconnaissance de sa cible, le complexe RISC inhibe l'expression de ce gène soit en clivant l'ARN ou en inhibant sa traduction (Figure 1.3, (IV))(Bologna et Voinnet, 2014; Elvira-Matelot et al., 2017).

1.4.1 Diversité, biosynthèse et fonction des sARNs

Chez les plantes, les sARNs ont différentes appellations selon le précurseur duquel ils sont produits. On distingue donc les miARNs et les siARNs.

1.4.1.1 miARNs

Les miARNs jouent un rôle crucial dans le contrôle de diverses voies cellulaires et participent à la plupart des processus biologiques et métaboliques chez les plantes et les animaux (Liu et al.,

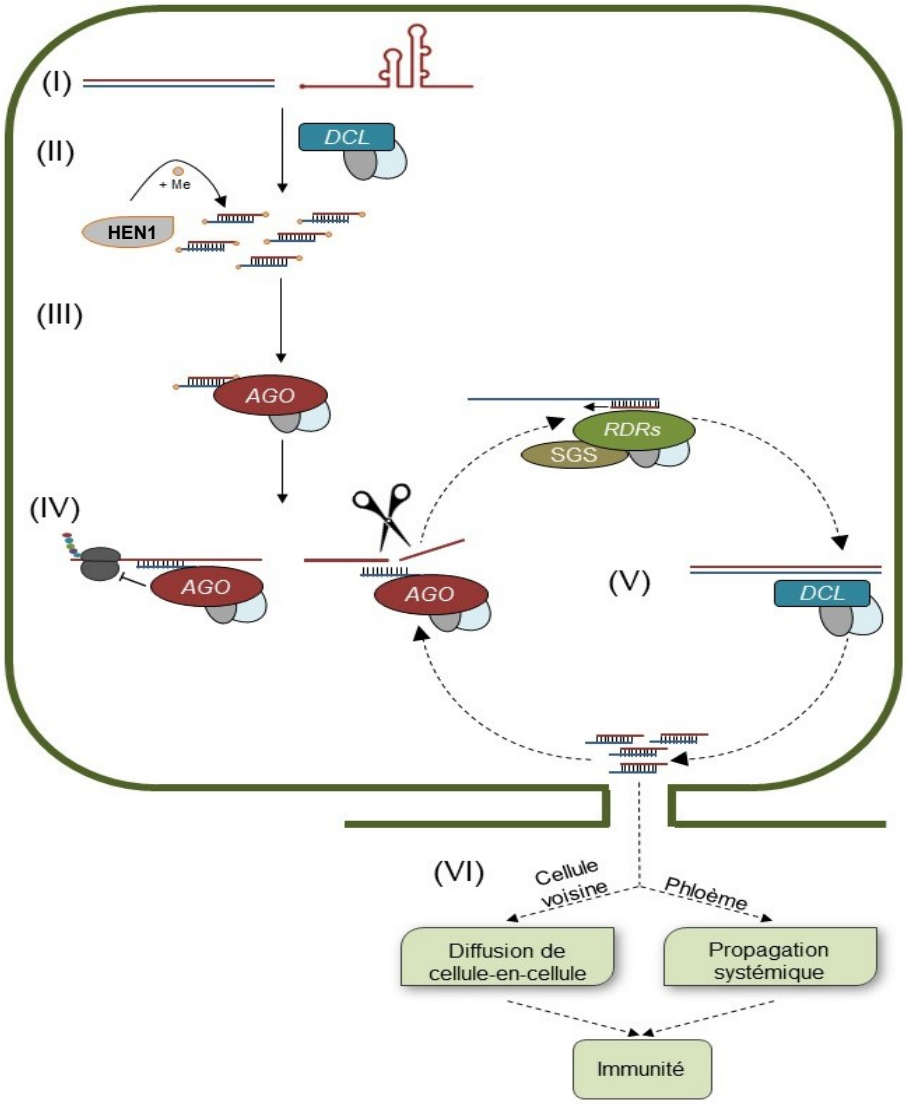


Figure 1.3 Mécanisme de l'ARN interférence.

(I) L'ARNdb dans le cytoplasme de la cellule (II) est reconnu et clivé par les protéines DCL menant à la production de sARN. (III) Suite à diverses étapes de maturation, les sARNs sont incorporés dans le complexe RISC composé, entre autres, d'une protéine AGO. (IV) RISC inhibera l'expression de tout acide nucléique présentant une complémentarité avec le sARN incorporé soit en inhibant sa traduction ou en le clivant. (V) Par la suite, il y aura production de novo d'ARNdb via l'action des protéines RDRs et SGS afin de permettre l'amplification du signal requis pour (VI) le mouvement de cellule-en-cellule et le mouvement systémique du signal, conférant l'immunité.

2017). La plupart des gènes miARN résident entre des gènes codant pour des protéines ou à l'intérieur des introns (Bartel et Bartel, 2003). Ils sont transcrits de façon indépendante les uns-des-autres et leur patron d'expression démontre une grande spécificité cellulaire et tissulaire. Leur production débute dans le noyau par leur transcription par l'ARN polymérase II (Pol II) en longs miARN primaires (pri-miARNs). Les pri-miARNs sont des molécules d'ARN monocaténaire polyadénylées qui se replient en structures en épingle à cheveux. Les pri-miARNs sont ensuite clivés en miARNs matures par la protéine DCL1 assistée par d'autres protéines tels que HYPONASTIC LEAVES 1 (HYL1) qui est une protéine liant l'ARNdb et SERRATE (SE) qui est une protéine à doigt de zinc (Reinhart et al., 2002; Kurihara et al., 2006; Dong et al., 2008). Ces miARNs sont exportés du noyau vers le cytoplasme de façon dépendante et indépendante de la protéine HASTY, une exportine-5 (Park et al., 2005). Comme n'importe quel sARNs, les miARNs matures sont aussi méthylés à leur extrémité 3' par HEN1 (Park et al., 2002). La majorité des miARNs sont incorporés dans la protéine AGO1.

Le brin guide microARN (miRNA) et son brin complémentaire (miRNA*) proviennent tous deux du duplexe miARN/miARN*. Généralement, les brins guides agissent comme des régulateurs post-transcriptionnels qui suppriment l'expression génique en clivant leurs transcrits d'ARNm cible, alors que les brins complémentaires étaient considérés comme dégradés en tant que brins passagers, mais il a été confirmé que le brin complémentaire possédait une fonctionnalité biologique significative (Liu et al., 2017).

Les miARNs/miARNs*, en plus de réguler finement le développement, jouent un rôle fondamental dans la réponse à divers stress abiotiques et biotiques (Liu et al., 2017). En effet, il est connu que l'ARN interférence joue un rôle important dans la défense contre les bactéries et les virus (Jin, 2008; Li et al., 2012). Des miARNs ont été observés pour réguler les ARNm impliqués dans la promotion de l'immunité des plantes dans les réponses PTI et ETI (Jin, 2008, Li et al., 2012; Fei et al., 2016). Pour donner un exemple, il a été démontré que l'association du miR393b* avec la protéine AGO2 permet d'assurer la résistance face à l'agent pathogène *Pseudomonas syringae* pv. Tomato (Pst) (Zhang et al., 2011). Le miR393b* cible le gène MEMB12, un SNARE localisé dans le Golgi, responsable du transport rétrograde du Golgi vers

le réticulum endoplasmique (RE) pour le recyclage des protéines et le maintien de l'équilibre. De cette façon, MEMB12 régule négativement l'exocytose de PR1, un composé antimicrobien. En inhibant la traduction de l'ARNm de MEMB12, l'association AGO2-miR393b* permet d'accroître la sécrétion de PR1. Dans le cas des virus, les miARNs les mieux caractérisés pour leur régulation de la défense antivirale sont probablement le miR168 et le miR403 ciblant respectivement AGO1 et AGO2, toutes deux impliquées dans la défense antivirale contre différents virus.

1.4.1.2 siARNs

Comme les miARNs, les siARNs sont une classe de sARNs jouant un rôle important dans le développement des plantes et la réponse contre divers stress chez les plantes. Il existe quatre classes de siARNs - les *heterochromatic* siARNs (hc-siARNs), les *trans-acting* siARNs (ta-siARNs), les *phased* siARNs (phasiARNs), les *natural antisens transcript* siARNs (nat-siARNs) - qui varient selon leur biogenèse (Axtell, 2013).

La voie des hc-siARNs. Les hc-siARNs, majoritairement d'une longueur de 24-nt, sont impliqués dans la voie de méthylation de l'ADN dépendante de l'ARN (RdDM) qui induit une répression de l'expression génique au niveau transcriptionnel (TGS). Les hc-siARNs sont dérivés de régions génomiques intergéniques et/ou répétitives et induisent des modifications répressives de la chromatine. La voie canonique simplifiée de leur biogenèse et de leur mode d'action débute par la transcription de ces loci dépendante de l'ARN polymérase IV (PolIV). Cet ARN simple brin (ARNsb) est copié en ARNdb par la polymérase à ARN dépendante de l'ARN 2 (RDR2) assisté par le remodeleur de la chromatine CLASSY1 (CLSY1). Cet ARNdb est clivé par DCL3 en siARNs d'une longueur de 24-nt qui sont méthylés puis incorporés majoritairement dans la protéine AGO4 ou les protéines faisant partie du clade AGO4, tel qu'AGO6 et AGO9 (Eun et al., 2011; Olmedo-Monfil et al., 2010). Il a été montré récemment qu'une partie de la population des hc-siARNs sont incorporés dans AGO3 (Zhang et al., 2016). Au cours de la phase effectrice, la PolV débutera la transcription des régions cibles de l'ADN (par exemple : régions promotrices, transposons, séquences répétées, etc). AGO4 (ou autres

AGOs ayant incorporé les hc-siARNs) sera rapidement recruté à ces loci par la PolV et d'autres facteurs ce qui permettra l'appariement, de façon séquence-spécifique, entre le hc-siARNs et l'ARN en cours de transcription. AGO4 recrute alors différentes protéines impliquées dans la méthylation de l'ADN ou l'ajout de marques répressives sur les queues d'histone (Matzke and Mosher, 2014). La reprogrammation de l'expression génique induite par cette voie est importante tant dans le développement normal que pour la réponse à différents stress biotiques et abiotiques (Coego et *al*., 2005; Agorio and Vera, 2007; López et *al*. 2011; Yu et *al*., 2013). Les hc-siARNs contribuent aussi à la défense antivirale contre les virus à ADN (Raja et al., 2008; Raja et al., 2014; Coursey et al., 2018).

La voie de ta-siRNAs. Les ta-siARNs, d'une taille de 21-nt, sont une classe de siARNs secondaires qui dérivent d'une voie bien spécialisée de l'ARN interférence où interviennent tant la voie de biosynthèse des miARNs que celle des siARNs. En effet, la voie des miARNs produit des miARNs spécifiques à la voie des ta-siARNs qui guident le clivage des ARNs précurseurs des ta-siARNs (Yoshikawa et al., 2005; Allen et al., 2005). La voie des ta-siARNs débute par la transcription d'ARN non-codant *TAS,* par la PolII. Ces transcrits sont dépourvus de structure secondaire extensive ce qui permet AGO1, ayant incorporé un miARN encodé par le génome, de clivé cet ARN. Les produits du clivage seront stabilisés par *SUPPRESSOR OF GENE SILENCING 3* (SGS3). Un des deux produits de clivage du transcrit *TAS* sera ensuite transformé en une matrice d'ARN double brin sous l'action de RDR6. Cette matrice d'ARNdb néoformée sera alors clivée en duplexes siRNAs de 21-nt par l'action de DCL4 et DRB4, et méthylé par HEN1 (Yoshikawa et al., 2005; Gasciolli et al., 2005; Adénot et al., 2006). Les ta-siARNs matures s'associeront majoritairement avec AGO1 (*TAS1, TAS2, TAS4*) et dans AGO7 (*TAS3*) pour guider le clivage d'ARNm complémentaire (Fahlgren et al., 2006, Montgomery et al., 2008). Cette voie de l'ARN interférence est impliquée dans le développement, l'homéostasie cellulaire et la régulation de l'auxine, une hormone. À ce jour, aucune étude n'a démontré l'implication de cette voie dans l'immunité végétale.

La voie des pha-siARNs. Cette voie intervient dans la transitivité du signal i.e. l'amplification du signal. La mécanistique de la production des pha-siARNs ressemble énormément à celle des

ta-siARNs. Cependant, dans celle-ci, il n'y a pas de miARNs qui entrent en jeu mais plutôt un siARN primaire, par exemple un siARN dérivé d'un virus (vsiARNs), qui va permettre le clivage d'un transcrit codant pour une protéine, par exemple Pentatricopeptide repeat (PPR), les facteurs de transcription MYB et les NB-LRR. Ceux-ci participent donc à la régulation de l'expression de ces gènes. Une autre différence majeure est l'implication de la protéine SILENCING DEFECTIVE 3 (SDE3) impliquée dans la production des pha-siARNs avec DCL4 et SGS3. Cette voie est aussi impliquée dans l'amplification du signal lors de la défense contre un virus, une étape cruciale pour éliminer l'agent pathogène. De par son rôle de régulateur de l'expression des PPR, NB-LRR et MYB qui sont tous impliqués dans l'immunité végétale, cette voie est d'une grande importance dans l'immunité végétale (Brodersen and Voinnet, 2006).

1.4.1.3 Stabilité des sARNs et de leurs précurseurs

La stabilité des sARNs et de leurs cibles est fortement régulée. Tel que mentionné précédemment, la méthylation des sARNs par HEN1 augmente significativement leur stabilité. De plus, le complexe RISC peut influencer la stabilité des sARNs. La plupart des miARNs endogènes sont incorporés dans un complexe RISC, et seulement une très petite proportion est libre dans les cellules. Il a été démontré que la délétion de certaines protéines AGO compromet la stabilité des sARNs. Par exemple, la perte de fonction de AGO1, l'effecteur majeur dans la voie des miARNs chez *Arabidopsis*, entraîne une réduction des niveaux de la plupart des miARNs (Vaucheret et al., 2006). *Arabidopsis* AGO2 est fortement induite par l'agent pathogène bactérien *Pseudomonas syringae*. Pendant ce temps, l'accumulation de plusieurs miRNA* qui sont liés par AGO2 est également élevée (Zhang et al., 2011). Curieusement, *Arabidopsis* AGO10 s'associe spécifiquement à miR165/166, mais l'accumulation de miR165/166 est augmentée, plutôt que réduite, chez les 10 mutants, ce qui implique que AGO10 réprime probablement le niveau de miR165/166 au lieu de stabiliser ce miARN (Liu et al., 2009; Zhu et al., 2011; Ji et al., 2011). Par conséquent, différentes protéines AGO pourraient affecter différemment la stabilité des petits ARN associés.

HEN1 SUPPRESSOR1 (HESO1) est l'un des 10 gènes connus de la famille des polymérases à ADN β chez *Arabidopsis*. HESO1 est capable d'ajouter une queue de nucléotides, préférentiellement des uridines, à l'extrémité 3' des sARNs non-méthylés par HEN1. Cette uridylation mène à la dégradation des sARNs et donc mène vers une régulation négative de l'ARN interférence. La persistance de courtes queues sur les sARNs dans les double mutants *hen1 heso1* suggère que d'autres nucléotidyltransférases terminales peuvent modifier les substrats des miARN en l'absence d'activité HESO1 (Ren et al. 2012; Zhao et al. 2012).

Chez *Arabidopsis*, une famille de NUCLÉASES DÉGRADANTES DE PETITS ARNs 1 (SDN1) a été identifiée de par sa similarité avec les exonucléases Rex de la levure. L'inactivation génique simultanée de plusieurs SDN entraîne une augmentation de sARNs matures suggérant que cette famille de protéine régule négativement la stabilité des sARNs et donc de la régulation négative du mécanisme de l'ARN interférence (Ramachandran et Chen, 2008). SDN1 possède aussi une activité exonucléase 3'- 5' contre des ARNsb courts mais n'est pas active contre des substrats plus long (Rogers and Chen, 2012).

Chez *Arabidopsis*, les *RNASE THREE-LIKE* RTL1 et RTL2 clivent les longs ARNdb et influencent l'accumulation et la fonction des sARNs. Les plantes surexprimant RTL1 démontrent une diminution dans l'accumulation de siARNs, mais pas dans l'accumulation de miARNs, conduisant à la conclusion que RTL1 dégrade des substrats d'ARNdb longs, parfaits ou presque parfaits (Shamandi et al., 2015). En revanche, RTL2 clive les substrats d'ARNdb en plus petites molécules d'ARNdb > 25-nt-long. Cette activité peut augmenter ou diminuer l'accumulation de différentes catégories de p4-siRNA endogènes PolIV et DCL3-dépendants qui affectent la méthylation du génome (Comella et al., 2016; Elvira-Matelot et al., 2016).

SKI2, SKI3 et SKI8, co-facteurs de l'exosome, possèdent un rôle direct dans l'inhibition de l'amplification du signal. En dégradant les fragments 5' issus du clivage initial induit par l'action d'un miARN, elles limitent la production de siRNAs secondaires (Branscheid et al., 2015).

On sait depuis longtemps que certaines DRBs sont essentielles à l'activité de clivage de certaines DCLs. Cependant, un nouveau clade de DRBs a été démontré récemment comme étant de potentiels inhibiteurs de la production de certains siARNs. En séquestrant l'ARNdb, ces protéines empêchent le clivage de ces substrats par les DCLs (Tschopp et al., 2017; Montavon et al., 2017).

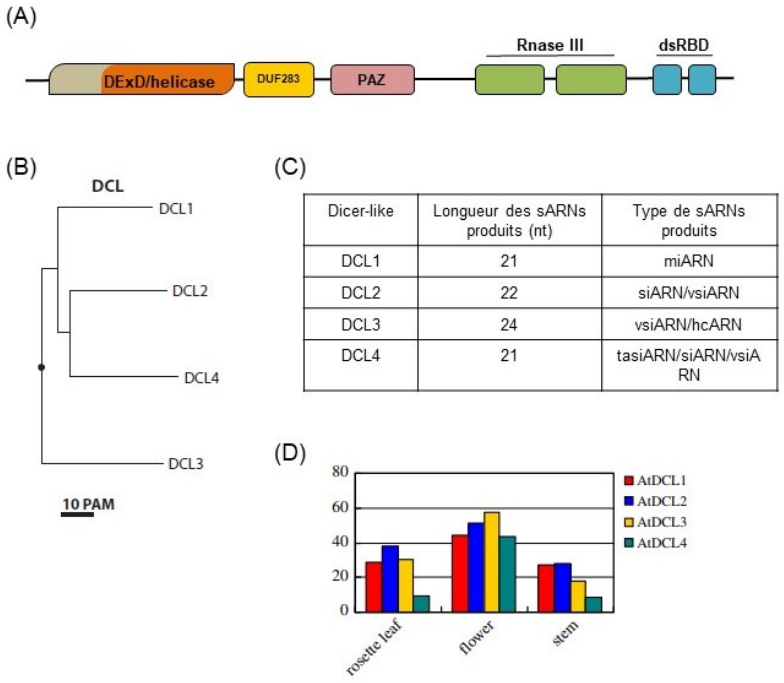
1.4.2 Principaux acteurs protéiques impliqués dans l'ARN interférence

1.4.2.1 Protéines Dicer-like (DCL)

Les enzymes responsables de la production de miARNs et siARNs à partir d'un intermédiaire l'ARNdb long appartiennent à la famille des DCL, des RNase de type III ayant une activité endonucléolytique. *Arabidopsis thaliana* et *Nicotiana benthamiana*, les plantes modèle utilisées au cours de mes projets de recherche, encodent quatre DCLs (DCL1, DCL2, DCL3 et DCL4) (Margis et al., 2006)(Figure 1.4(B)).

Ces quatre DCLs présente une structure de base similaire soit, un domaine DExD-box/helicase, un domaine DUF283, un domaine PAZ et un domaine de liaison à l'ARNdb (dsDRB) (Bologna et Voinnet, 2014)(Figure 1.4(A)). Le nombre de ces domaines varient d'une DCL à l'autre. Par exemple, DCL1, DCL3 et DCL4 possèdent deux domaines dsDRB mais DCL2 n'en possède qu'un. Le domaine DExD-box/helicase possède généralement une activité hélicase et permettrait le déplacement des protéines le long de l'ARN pour permettre les coupes successives. Ils peuvent également fonctionner comme plateforme d'assemblage pour les gros complexes ribonucléique et agirait aussi comme capteurs de l'ATP, qui est requis pour le clivage (Margis et al., 2006). Le domaine DUF283 serait un domaine permettant de lier l'ARN (Qiu et al., 2010). Le domaine PAZ aurait un role dans la determination de la longueur spécifique des sARNs produits par chacune des DCLs (Margis et al., 2006) (Figure 1.4(C)).

Outre leurs similitudes au point de vue structural, les DCL végétales jouent différents rôles dans



Dicer-like	Longueur des sARNs produits (nt)	Type de sARNs produits
DCL1	21	miARN
DCL2	22	siARN/vsiARN
DCL3	24	vsiARN/hcARN
DCL4	21	tasiARN/siARN/vsiARN

Figure 1.4 Les DCLs chez *Arabidopsis thaliana*.

(A) Schématisation des domaines des DCLs. (B) Arbre phylogénétique des DCLs (Tiré de Bologna et Voinnet, 2014). (C) Description des sARNs produits par les DCL. (D) Patron d'expression des DCLs (Tiré de Liu et al., 2009).

le développement des plantes et au cours de la réponse à divers stress. En effet, les DCLs ne sont pas uniformément exprimés dans les tissus, à différents stades de développement et en réponse aux stress environnementaux (Liu et al., 2009)(Figure 1.4(D)).

DCL1. DCL1 est l'enzyme responsable de la synthèse de la très grande majorité des miARNs et possède donc un rôle fondamental dans le développement. De par son rôle crucial, la délétion de DCL1 est létale. Le second dsRBD C-terminal, présent dans DCL1, DCL3 et DCL4 mais absent dans DCL2, favorise la localisation du DCL1 dans le noyau, où il se rassemble avec d'autres facteurs de biogenèse des miARNs (Burdisso et al., 2012). Tel que mentionné précédemment, DCL1 nécessite DRB1 et, dans des tissus spécifiques, DRB2 pour la production efficace et précise des miARNs (Kurihara et Watanabe, 2004). Les protéines, HYL et SE, assistent DCL pendant la production des miARNs (Kurihara et al., 2005; Dong et al., 2008).

Finalement, DCL1 aurait des effets tant négatifs que positifs sur la réponse antivirale : elle serait impliquée indirectement dans la régulation négative de DCL4, importante pour la défense antivirale (Qu et al., 2008). Inversement, elle permettrait la génération de sARN dérivés de certains virus à ADN tel que les Caulimovirus et Geminivirus (Blevins et al., 2006).

DCL2 et DCL4. Ces deux DCLs seront traitées dans la même section puisqu'elles agissent de façon redondante (Parent et al., 2015) ou parfois hiérarchique (Deleris et al., 2006). DCL2 est impliquée dans la production des siARNs de 22-nt alors que DCL4 produit des siARNs de 21-nt. L'action de DCL4 requière l'assistance de DRB4 alors qu'il est encore incertain si l'action de DCL2 requière une DRB. Une fonction spécifique à DCL4 est dans la voie de génération des ta-siARNs. Cependant, ces deux protéines, DCL2 et DCL4, sont toutes les deux impliquées dans l'amplification du signal lors d'une réponse antivirale. En effet, on note une forte présence de vsiARNs de 21-nt et 22-nt lors d'une infection par un virus à ARN. La présence de vsiARNs de 22-nt est augmentée chez les plants mutants *dcl4* infectés suggérant ainsi une redondance mais aussi une hiérarchie dans la fonction des protéines DCLs, où DCL4 aurait un rôle antiviral prédominant et ce, tant dans les feuilles inoculées que dans les feuilles non-inoculées où elle préviendrait l'infection systémique en empêchant la sortie des virus du système vasculaire. DCL2 aurait quant à elle une activité antivirale accessoire et interviendrait plus lorsque l'activité de DCL4 est supprimée (Deleris et al., 2006). Lorsqu'il y a un faible inoculum viral ou lors d'une infection avec un virus muté pour son suppresseur de l'ARN interférence, DCL2 serait suffisante pour empêcher l'infection systémique les virus TRV (Tobacco rattle virus) et TCV (Turnip crinkle virus).

DCL3. L'action de DCL3 sur les longues matrices d'ARNdb mène à la production des hc-siARNs, d'une longueur de 24-nt, impliqués dans la voie RdDM (Eun et al., 2011; Olmedo-Monfil et al., 2010; Matzke and Mosher, 2014; Zhang et al., 2016). Tel que mentionné précédemment, les hc-siARNs permettent de contrôler l'expression génique au niveau de la transcription et permettent une défense efficace contre les virus à ADN. De plus, chez un double mutant dcl2 dcl4, les deux principales DCL antivirale contre les virus à ARN, on note une

production de vsiARNs d'une longueur de 24-nt suggérant que cette protéine peut accessoirement prendre le relais contre les virus à ARN (Bouché et al., 2006).

1.4.2.2 Protéines Argonaute (AGO)

Une fois produits, les duplexes sARNs, peu importe leur nature, sont incorporés dans le complexe effecteur RISC. Le complexe RISC est un holocomplexe dont la composition est encore peu connue. Il est, entre autres, composé du sARN ainsi que d'une protéine de la famille des ARGONAUTES (AGO). Cependant, plusieurs études démontrent que les protéines capables d'interagir avec les protéines AGOs ont des motifs répétés de Glycine/tryptophane (WG/GW) (Till et al., 2007). Contrairement à d'autres modèles, on connaît seulement un nombre limité de protéine pouvant interagir avec les AGOs végétales. Jusqu'à présent, les interacteurs connus inclut : la cyclophiline40 (CYP40); une protéine de choc thermique (HSP90), la transportine1 (TRN1) qui interagissent avec AGO1 pour faciliter le chargement des miARNs (Iki et al., 2009; Smith et al., 2009; Iki et al., 2010; Cui et al., 2016) alors que la PolV, SPT5 (un facteur d'élongation de la transcription) et WGRP1 (une oxidoréductase putative) intéragissent avec AGO4 (El-Shami et al., 2007; Bies-Etheve et al., 2009; He et al., 2009; Karlowski et al., 2010). Puisque les protéines AGO sont les protéines effectrices indispensables du complexe RISC, la structure et les fonctions de ces dernières seront détaillées dans les prochaines sections.

Le nombre de protéines AGOs encodées varie beaucoup entre les organismes. La plante modèle, *Arabidopsis*, encode dix protéines AGOs que l'on peut diviser en trois clades distincts. Le clade I (AGO1-Clade) inclut AtAGO1/AtAGO5/AtAGO10; le clade II (AGO2-clade) inclut AtAGO2/AtAGO3/AtAGO7 et finalement le clade III (AGO4-Clade) inclut AtAGO4/AtAGO6/AtAGO8/AtAGO9. Cependant, bien que certains membres d'un même clade présentent des redondances fonctionnelles, comme AtAGO1 et AtAGO10, cette classification est basée sur une homologie de séquence et n'indique pas nécessairement qu'elles ont des fonctions similaires (Mallory et al., 2009; Mallory et Vaucheret, 2010).

1.4.2.2.1 Domaines fonctionnels des AGO

Les AGO eucaryotes canoniques contiennent quatre domaines principaux: un domaine N-terminal variable et les domaines PAZ, MID et PIWI plus hautement conservés qui, ensemble, positionnent correctement les sARNs par rapport à leurs cibles (Figure 1.5).

Étant donné le faible niveau d'homologie entre les domaines N-terminal des différentes AGOs, ce domaine a longtemps été ignoré lors des études. Chez un modèle animal, Bas Kwak et Tomari ont démontré l'importance de ce domaine chez Ago2 pour la maturation du complexe RISC, i.e. la séparation du brin guide et du brin passager (Bas Kwak and Tomari, 2012). Cependant, une telle fonction du domaine N-terminal n'a pas été démontré chez les AGO végétales et le rôle du domaine N-terminal n'est toujours pas élucider. Récemment, le Block43, un motif répété et conservé chez certaines AGO animales et végétales a été identifié (Rodriguez-Leal et al., 2016). Néanmoins, la fonction et l'importance de ce bloc n'ont pas encore été élucider.

Le domaine MID lie l'extrémité 5' du sARN et possède une boucle de spécificité nucléotidique permettant de reconnaître spécifiquement le nucléotide 5 'des sARNs. Cette spécifité diffère entre les AGOs (Frank et al, 2012). Par exemple, AGO1 lie préférentiellement les miARNs ayant un 5'U, AGO2 lie préférentiellement les sARNs ayant un 5'A, AGO3 lie préférentiellement les 5'A, AGO5 lie préférentiellement les sARNs ayant un 5'C et AGO4 lie préférentiellement les hc-siARNs ayant un 5'A (Mi et al., 2008, Montgomery et al., 2008; Takeda et al., 2008; Zhang et al., 2016). Le domaine PAZ est responsable de la liaison de l'extrémité 3' des sARNs. Chez les protéines AGO humaines, le domaine PAZ contribue également au déroulement des sARNs pendant la maturation du complexe RISC (Gu et al., 2012). Finalement, ce domaine est responsable de la spécificité de longueur des sARNs incorporés dans l'AGO. Par exemple, AGO1 s'associe préférentiellement au sARNs d'une longueur de 21- ou 22-nt; AGO2, des sARNs de 21-nt alors qu'AGO3, AGO4, AGO6 et AGO9 lie préférentiellement les sARNs de 24-nt (Mi et al., 2008, Montgomery et al., 2008; Takeda et al., 2008; Zhang et al., 2016). Le domaine PIWI adopte un repliement de type RNase-H et présente une activité d'endonucléase médiée par une triade catalytique Asp-Asp-His (DDH) ou

Asp-Asp-Asp (DDD) chez AGO2 et AGO3 (Carbonell et al., 2.012). Avec cette activité catalytique, le domaine PAZ facilite la séparation du duplexe sARN par le clivage du brin passager du sARN lorsque les brins possèdent une complémentarité extensive. Ce domaine est aussi responsable du clivage de l'ARN cible de façon séquence-spécifique au brin guide incorporé (Bas Kwak and Tomari, 2012).

1.4.2.2.2 Description des fonctions des AGOs

1.4.2.2.2.1 Clade AGO1 (AGO1, AGO10, AGO10)

AGO1. AGO1 est impliqué dans la régulation de divers endogènes via son association majoritairement avec des miARNs (Vaucheret et al., 2004). AGO1 est considéré comme l'effecteur des miARNs et des ta-siRNA (Vaucheret et al 2004, Baumberger et Baulcombe 2005, Qi et al 2005, Mi et al 2008). Ces miRNAs et ta-siRNAs guident AGO1 pour réguler la stabilité et/ou la traduction d'ARNm de gènes impliqués dans de nombreux processus développementaux et physiologiques. La régulation de la plupart des cibles endogènes par AGO1 requière son activité catalytique de clivage (Arribas-Hernandez et al., 2016). Cependant, AGO1 peut aussi réprimer l'expression de ces cibles en inhibant leur traduction. Pour accomplir cette fonction, AGO1 requière l'assistance d'une protéine associée au ER soit ALTERED MERISTEM PROGRAM1 (AMP1). Les ARNs dont leur traduction est inhibée se trouvent moins associés aux polysomes (Li et al., 2015). Finalement, une étude très récente a montré qu'AGO1 est requis pour le transfert des miARNs du noyau vers le cytoplasme (Bologna et al., 2018). AGO1 intervient également dans la défense antivirale en s'associant avec des vsiARNs de 21- et 22-nt produits par DCL4 et DCL2, respectivement (Morel et al., 2002; Qu et al., 2008; Wang et al., 2011)(Tableau 1).

AGO10. AGO10 est l'homologue le plus proche d'AGO1 au niveau de la séquence. Cependant, leur patron d'expression et leurs fonctions dans le développement diffèrent (Vaucheret, 2008). AGO10 joue un rôle dans la régulation de l'expression génique via le clivage ou l'inhibition de la traduction de ses cibles. AGO10 est impliqué dans l'inhibition de la traduction d'AGO1 via

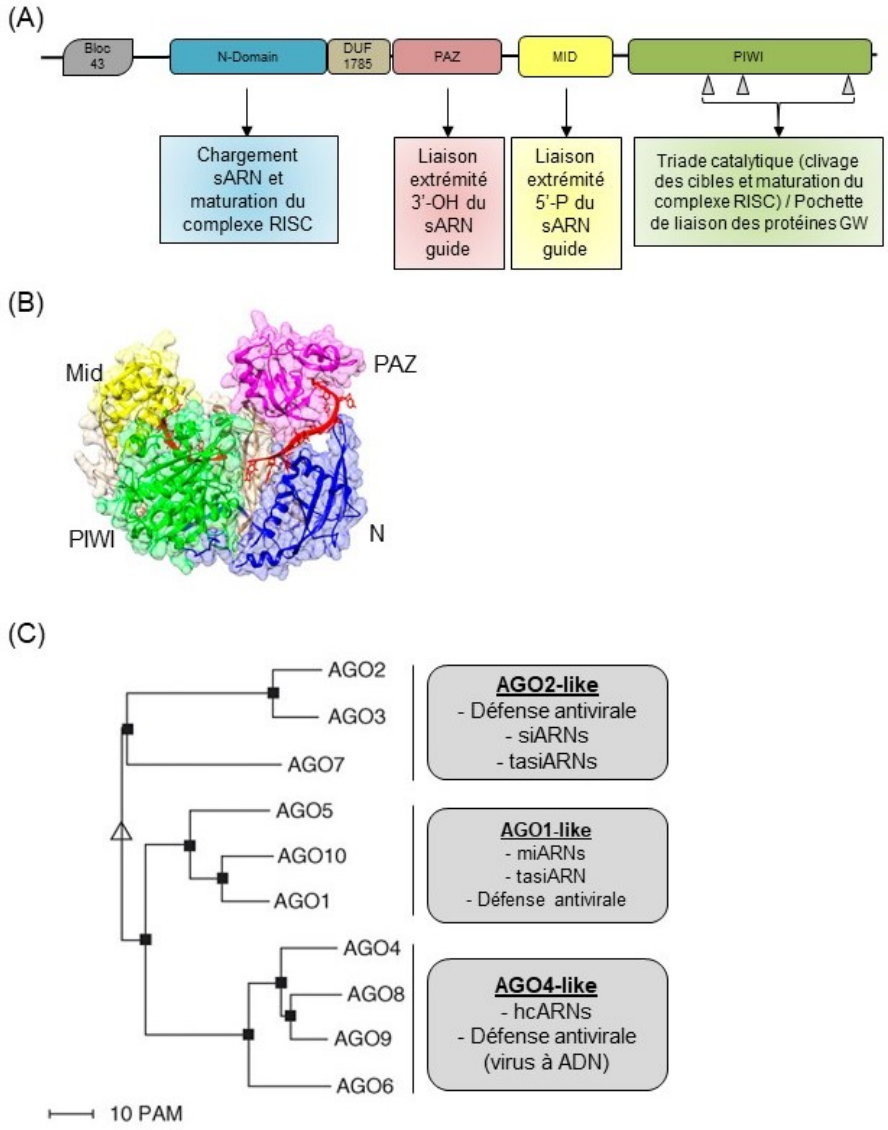


Figure 1.5 Les AGOs chez *Arabidopsis thaliana*.

(A) Schématisation des domaines fonctionnels des AGOs. (B) Structure 3D des protéines AGOs (C) Arbre phylogénétique des AGO chez *Arabidopsis* et leurs fonctions (Tiré de Vaucheret, 2008).

le miR168 (Vaucheret et al., 2006; Mallory et al., 2009). Une étude de 2011 a démontré qu'AGO10 régule le développement du méristème apical en séquestrant les miR166/165 empêchant ainsi leur incorporation dans AGO1, la protéine effectrice pour ces miARNs (Zhu et

al., 2011). AGO10 possède une activité antivirale très faible contre le Turnip mosaic virus (TuMV) (Garcia-Ruiz et al., 2015)(Tableau 1).

AGO5. Chez le riz et *Arabidopsis*, AGO5 est exprimé dans les tissus reproducteurs tel que les tissus avoisinants les mégaspores, les mégaspores et dans le cytoplasme du pollen mature (Schmid et al., 2008; Kapoor et al., 2008; Tucker et al., 2012). Étant donné son homologie avec AGO1, on suppose qu'il peut diriger les fonctions dirigées par les miARNs et les siARNs. En 2015, un groupe a démonté qu'AGO5 joue un rôle accessoire dans la défense contre TuMV (Garcia-Ruiz et al., 2015)(Tableau 1).

1.4.2.2.2.2 Clade AGO2 (AGO2, AGO3, AGO7)

AGO2. AGO2 possède plusieurs fonctions. Elle régule l'expression génique, participe à la réparation de l'ADN suite à un stress génotoxique, induit la méthylation de certains loci et est impliquée dans la défense antibactérienne et antivirale (Maunoury et Vaucheret, 2011; Wei et al., 2012; Pontier et al., 2012; Zhang et al., 2015; Harvey et al., 2011; Wang et al., 2011; Zhang et al., 2012; Ma et al., 2015; Jaubert et al., 2011; Carbonell et al., 2012; Scholthof et al., 2011; Alazem et al., 2016)(Tableau 1).

AGO3. En dépit de sa proximité phylogénétique et génomique avec AGO2, très peu de fonction ont été attribué à AGO3. Récemment, il a été montré qu'AGO3 participe, de façon partiellement redondante à AGO4, à la TGS via les hc-siARNs (Zhang et al., 2016). Une seule étude a démontré l'implication d'AGO3 dans la défense antivirale (Alazem et al., 2016). L'induction de l'expression d'AGO3 lors d'une infection par le Bamboo mosaic virus (BaMV) est dépendante de l'acide abscissique (ABA)(Tableau 1).

AGO7. AGO7 est responsable de la régulation de l'expression génique via les TAS3 ta-siARNs. Cette famille de ta-siARNs sont connus pour cibler plusieurs gènes répondant à l'auxine (ARF) qui sont impliqués dans la régulation du développement des organes latéraux et de la

synchronisation du développement (Fahlgren et al., 2006, Montgomery et al., 2008). AGO7 possède aussi un rôle antiviral mais seulement contre un TCV atténué (Qu et al., 2008).

1.4.2.2.2.3 Clade AGO4 (AGO4, AGO6, AGO8, AGO9)

AGO4. AGO4 est l'effecteur principal de la RdDM et de la TGS via son association avec les hc-siARNs. De façon intéressante, il a été montré qu'AGO4 charge les hc-siARNs dans le cytoplasme des cellules. Le chargement des hc-siARNs induit probablement un changement de conformation de la protéine ce qui permet de démasque un signal de localisation nucléaire (NLS)(Ye et al., 2012). Comme les autres AGOs, AGO4 possède aussi la triade catalytique dans le domaine PIWI. Il a été démontré que cette fonction est requise pour la méthylation de l'ADN à certains loci (Qi et al., 2006). AGO4 joue aussi un rôle dans la défense antibactérienne et contre les virus à ADN et ARN (Zhang et al., 2011; Raja et al., 2008; Raja et al., 2014; Ma et al., 2015)(Tableau 1).

AGO6. AGO6 possède un rôle partiellement redondant à celui d'AGO4 dans la méthylation de l'ADN et la TGS (Zheng et al., 2007). AGO6 est impliqué dans la RdDM seulement dans un sous-ensemble spécifique de tissus, tel que les tissus méristématiques de la tige et des racines et affectant seulement certains loci (Eun et al., 2011). Ceci est en accord avec son patron d'expression qui se limite aux mêmes tissus.

AGO8. AGO8 a longtemps considéré comme un pseudogène (Takeda et al., 2008). Néanmoins, une étude récente a démontré qu'AGO8 module plusieurs nœuds régulateurs dans le réseau de signalisation au cours de la réponse aux herbivores chez *Nicotiana attenuata*. Dans cette étude, ils ont montré que cette réponse dépendait aussi de RDR1, DCL3 et DCL4 (Pradhan et al., 2017).

AGO9. AGO9 est exprimé dans les ovules, les anthères et les téguments de la graine où elle contrôle la formation de gamètes femelles (Olmedo-Monfil et al., 2010). AGO9 est aussi impliquée dans l'inhibition des transposons dans les gamètes femelles et les cellules accessoires.

Lorsqu'exprimé sous le contrôle du promoteur d'AGO4, AGO9 arrive à partiellement restaurer la RdDM.

Les AGOs ont différentes façons de compromettre l'infection par les virus. Évidemment, le mécanisme le plus évident est une interférence directe avec l'ARN viral, soit en le clivant ou en inhibant sa traduction. Dans ce scénario, une AGO incorpore des vsiARNs (Figure 1.6(A)). Cependant, une AGO peut aussi induire la défense antivirale en modulant l'expression génique. Certaines classes de sARNs sont induits par les infections virales. Ce sont les vasiRNAs, pour *viral activated siRNAs*. Ils sont majoritairement produits par l'action de DLC4 et RDR1 et incorporés dans AGO2 (Cao et al., 2014). Dans ce scénario, des sARNs endogènes sont incorporés dans AGO2. La répression de ces gènes permet de monter une réponse de défense antivirale (Figure 1.6(B)).

1.4.2.2.3 Régulation des AGO

Jusqu'à maintenant, seulement trois protéines AGOs ont été démontrées pour être régulées de façon post-transcriptionnelle par la voie de l'interférence à l'ARN. AtAGO1 est ciblée par le miR168 (Rhoades et *al.*, 2002; Vaucheret et al., 2006) alors qu'AGO2 et AGO3 sont ciblées par le miR403 (Allen et *al.*, 2005). La régulation de l'expression d'AGO1, via le miR168 et les siARN dérivés d'AGO1, est dépendante d'AGO1 et AtAGO10 par une boucle de rétroaction négative, où AGO1 agit par clivage et AGO10 agit par inhibition de la traduction (Mallory et *al*., 2009). En ce qui concerne la régulation d'AGO2 et AGO3, la régulation post-transcriptionnelle s'effectue par le chargement du mi403 dans la protéine AGO1 (Allen et *al.*, 2005). Le site ciblé par le miR a été identifié au sein du 3'UTR (Région non traduite) du transcrit d'AGO2 et AGO3. Ce site est conservé chez plusieurs familles de dicotylédones mais pas chez les orthologues de monocotylédones (Allen et *al.*, 2005).

Chez *Arabidopsis*, une étude a démontré que la région 3' UTR d'AGO10 contient des sites consensus de liaisons des protéines PUMILIO (APUM). Des essais in vitro ont permis de

Tableau 1. Liste des protéines AGOs ayant un rôle antiviral confirmé ou putatif (basé sur leur capacité de lier des vsiARNs ou sur leur capacité à cliver les ARN viraux *in vitro*). En gris : virus à génome à ADN.

Protéine	Virus	Hôte	Référence
Protéine dont l'action antivirale a été démontrée suite à l'invalidation génique.			
AGO1	CMV	*A. thaliana*	Morel et al., 2002 Wang et al., 2011
	TCV	*A. thaliana*	Qu et al., 2008
	BMV	*A. thaliana*	Dzianott et al., 2012
	ToRSV	*N. benthamiana*	Ghoshal et Sansfacon, 2014
	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015
AGO2	CMV	*A. thaliana*	Wang et al., 2011 Harvey et al., 2011
	PVX	*A. thaliana*	Jaubert et al., 2011
	TBSV	*N. benthamiana*	Scholthof et al., 2011
	TCV	*A. thaliana*	Harvey et al., 2011 Zhang et al., 2012
	TuMV	*A. thaliana*	Carbonell et al., 2012 Garcia-Ruiz et al., 2015
	TRV	*A. thaliana*	Ma et al., 2015
	BaMV	*A. thaliana*	Alazem et al., 2017
AGO3	BaMV	*A. thaliana*	Alazem et al., 2017
AGO4	BTCV	*A. thaliana*	Raja et al., 2008
	PVX	*N. benthamiana*	Bhattacharjee et al., 2009
	CaMV	*A. thaliana*	Raja et al., 2014
	TRV	*A. thaliana*	Ma et al., 2015
AGO5	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015
AGO7	TCV	*A. thaliana*	Qu et al., 2008
	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015
AGO10	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015
Protéine	Virus	Hôte	Référence
Protéine dont la capacité de lier les vsiARNs *in vivo* a été démontrée.			
AGO1	CMV	*A. thaliana*	Wang et al., 2011
	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015
AGO2	CMV	*A. thaliana*	Takeda et al., 2008 Wang et al., 2011
	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015
AGO4	CMV	*A. thaliana*	Hamera et al., 2012
AGO5	CMV	*A. thaliana*	Takeda et al., 2008
AGO10	TuMV	*A. thaliana*	Garcia-Ruiz et al., 2015

Tableau 1 (suite)

Protéine	Virus	Hôte	Référence
Protéine dont la capacité de clivage de l'ARN viral a été démontré *in vitro*.			
AGO1	TBSV	*A. thaliana*	Schuck et al., 2013
	TBSV	*N. tabacum*	Schuck et al., 2013
AGO2	TBSV	*A. thaliana*	Schuck et al., 2013
AGO3	TBSV	*A. thaliana*	Schuck et al., 2013
AGO5	TBSV	*A. thaliana*	Schuck et al., 2013

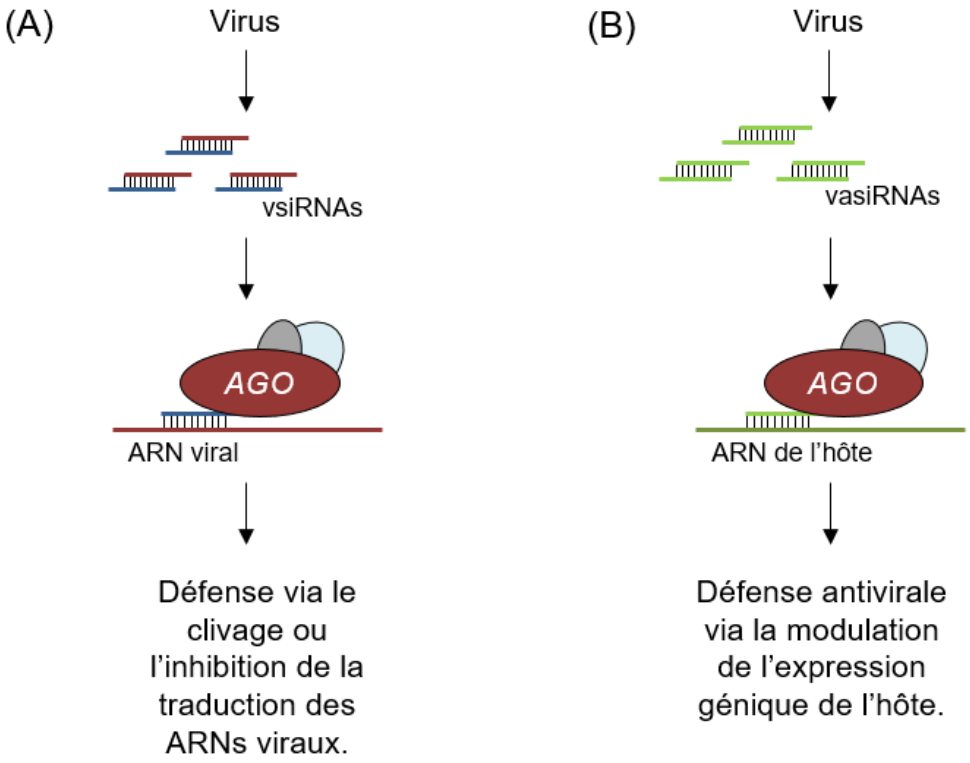


Figure 1.6 Mode d'action des AGOs antivirales.

(A) La voie classique où les vsiARNs servent à guider les AGO vers les vARNs. (B) La voie alternative au cours de laquelle la modulation de l'expression géniques par les vasiARNs permet la mise en place d'une réponse de défense.

conclure que les PUMILIO régulaient négativement l'expression d'AGO10 via son 3'UTR. Ils n'ont cependant pas démontré s'il s'agissait d'une déstabilisation de l'ARN ou l'inhibition de la traduction (Francishini & Quaggio, 2009). Aucune autre protéine impliquée dans l'interférence à l'ARN n'a été démontrée comme étant une cible potentielle de cette famille de protéine.

Au niveau post-traductionnel, i.e. au niveau de la stabilité/activité des protéines, peu de détails sont connus sur les régulateurs de la stabilité de ces protéines chez les plantes. Il est bien décrit dans la littérature qu'une protéine AGO est plus stable lorsqu'elle est associée à un sARN. Chez *Arabidopsis*, la stabilité d'AGO1 est compromise par FBW2, une F-BOX endogène (Earley et *al.*, 2010) et par l'autophagie lors d'une réponse de défense (Derrien et *al.*, 2012).

Chez les mammifères, la stabilité, la localisation et l'activité des AGOs sont finement régulées par des modifications post-traductionnelles. Par exemple, pour n'en nommer que quelques-unes, la prolyl 4-hydroxylation sur la P700 d'Ago2 déstabilise la protéine (Qi et al., 2008). Une phosphorylation sur la S387 d'Ago2 faciliterait sa relocalisation vers les *Processing bodies* (P-bodies)(Zeng et al., 2008).

Finalement, comme nous verrons plus loin, de nombreux suppresseurs de l'interférence à l'ARN exprimés par les virus (VSR) et les bactéries compromettent l'activité ou la stabilité des AGOs.

1.4.2.2.4 Mode d'action des AGOs dans la voie PTGS

1.4.2.2.4.1 Clivage de l'ARN

Tel que mentionné précédemment, la fonction catalytique des AGOs, Mg^{2+} -dépendante, est conférée par la triade catalytique DDH ressemblant au domaine des RNaseH des bactéries (Song et al., 2004; Schwarz et al., 2004). Certaines études ont démontré que des mutations dans cette triade sont délétères pour la fonction catalytique des AGO (Qi et al., 2005; Qi et al., 2006). Cependant, la présence du motif n'implique pas nécessairement une activité de clivage. En effet,

malgré qu'Ago3 chez l'humain présente un domaine DDH, la protéine ne semble pourtant pas posséder la capacité de cliver ses cibles (Liu et al., 2003; Meister et al., 2004). Ceci suggère donc que la présence du domaine PIWI (ou de la triade) en soit n'est pas suffisante pour induire une activité de clivage. Le remplacement des domaines MID-PIWI d'At-AGO1 par ceux d'AtAGO10, ayant aussi un domaine DDH, ne permet pas de restaurer l'interférence à l'ARN chez un mutant hypomorphique *ago1* (Mallory et al., 2009) suggérant encore une fois que d'autres propriétés des AGO sont requises pour induire l'activité de clivage. Inversement, l'absence de la triade catalytique n'exclut pas la possibilité d'une activité de clivage. Parmi les protéines AGO chez Arabidopsis, deux d'entre elles présentent un domaine catalytique dégénéré (DDD) soit AtAGO2 et AtAGO3. Malgré l'absence d'une triade catalytique conventionnelle chez AtAGO2, cette protéine possède tout de même une activité catalytique in vitro (Carbonell et al., 2012).

Les miARNs reconnaissent généralement leurs cibles principalement via une région limitée - appelée *seed region* - au niveau des nucléotides 2-7 ou 8 du brin guide miARN (Bartel, 2009). Cette région doit être parfaitement complémentaire à la cible pour permettre le clivage. Les protéines de l'argonaute impliquées dans la voie des miARN chez les animaux s'associent aux protéines GW182 qui jouent des rôles importants dans la répression de la désadénylation et de la traduction (Braun et al., 2012).

1.4.2.2.4.2 Inhibition de la traduction

Les protéines AGOs peuvent aussi réprimer l'expression génique de façon indépendante au clivage de la cible. Dans ce cas, l'ARNm de la cible sera toujours détectable mais pas la protéine pour lequel il code. Chez les *Arabidopsis*, AGO1, AGO2 et AGO10 possèdent cette capacité de d'inhiber la traduction de certaines de leurs cibles (Brodersen et al., 2008; Lanet et al., 2009; Zhang et al., 2011). Chez *Nicotiana benthamiana*, AGO4 est requise lors de la répression de la traduction des ARNs viraux (vARNs) lors d'une réponse ETI enclenchée par la reconnaissance de la P50 par le gène N (Bhattacharjee et al., 2009). Cependant, dans le dernier cas, on ne sait

pas si AGO4 agit elle-même directement sur les vARNs pour réprimer leur traduction ou si son action est indirecte.

Chez l'humain, l'inhibition de la traduction via les sARNs requiert l'expression de la protéine GW182 (Eulalio et al., 2005). Cette protéine permettrait l'interaction avec les facteurs de déadénylation qui agiraient en premier sur l'ARN cible suivi par les protéines décoiffantes. Cependant, malgré que plusieurs modèles animaux possèdent une ou plusieurs protéines GW182, aucun homologue n'a été identifié chez les végétaux. De plus, chez *Arabidopsis*, il a été montré que la répression de la traduction par AGO1 n'induit pas nécessairement la déadénylation, ni la dégradation de l'ARN cible (Iwakawa and Tomari, 2013). Les ARNs dont la traduction est inhibée sont parfois stocké dans différents foci cytoplasmiques soit, les *stress granules* (SG) ou les P-bodies. La composition protéique de ces deux types de granules est très différente. Des ARNs entrant dans les SG peuvent retourner en traduction active si les conditions changent alors que si les ARNs sont envoyés dans les P-bodies, ils seront décoiffés, déadénylés et dégradé (Decker et Parker, 2012).

1.4.3 Protéines ARN polymérase dépendante de l'ARN (RDR)

Le signal d'extinction, sARNs, généré par les protéines DCLs peut se disperser de cellule-en-cellule ou encore de façon systémique. Ce mouvement du signal nécessite une amplification basée sur la conversion de l'ARN cible en une nouvelle matrice d'ARN db (Figure 1.3 (V) (VI)). Cette action peut être accomplie par certaines ARN polymérases dépendantes de l'ARN (RDRs) de la plante (Dunoyer et al., 2003; Schwach et al., 2005). La structure des RDRs est définie par un domaine catalytique conservé requis pour la copie de l'ARN simple brin ARNsb en ARNdb et d'un domaine dsRBD(Bologna et Voinnet, 2014). Il existe trois clades majeurs de RDR eucaryotes: RDRα, RDRβ et RDRγ. Alors que RDRα est présent dans les plantes, les animaux et les champignons, RDRβ est unique aux animaux et aux champignons, alors que RDRγ est présent dans les plantes et les champignons (Wassenegger et Krczal, 2006)(Figure 1.7). Jusqu'à présent, il n'y a pas de rôle assigné aux RDRs du groupe RDRγ. Les RDRs les mieux

caractérisées chez les végétaux sont RDR1, RDR2 et RDR6 du groupe α. RDR2 permet de copier l'ARNsb, généré par la PolIV, en ARNdb qui sera reconnu et clivé par DCL3 pour générer des hc-siARNs impliqués dans la RdDM (Matzke and Mosher, 2014) alors que RDR6 est impliqué dans la production des ta-siARNs.

L'inactivation génique de RDR1, RDR2 et RDR6 augmente la susceptibilité tant aux virus à ARN que les virus à ADN (Pumplin et Voinnet, 2013). Parmi c'est trois RDRs, RDR1 et RDR6 sont les plus importantes pour monter une défense antivirale efficace. Les membres du clade RDRα ont également des rôles indirects dans la défense contre les agents pathogènes non-viraux (par exemple les bactéries, les oomycètes et les nématodes) et les herbivores en produisant des ARNs régulateurs endogènes, y compris des tasiRNA et des ARNsi antisens naturels (ARNsi nat)(Katiyar-Agarwal et al., 2006; Pandey et Baldwin, 2007; Hewei et al., 2010).

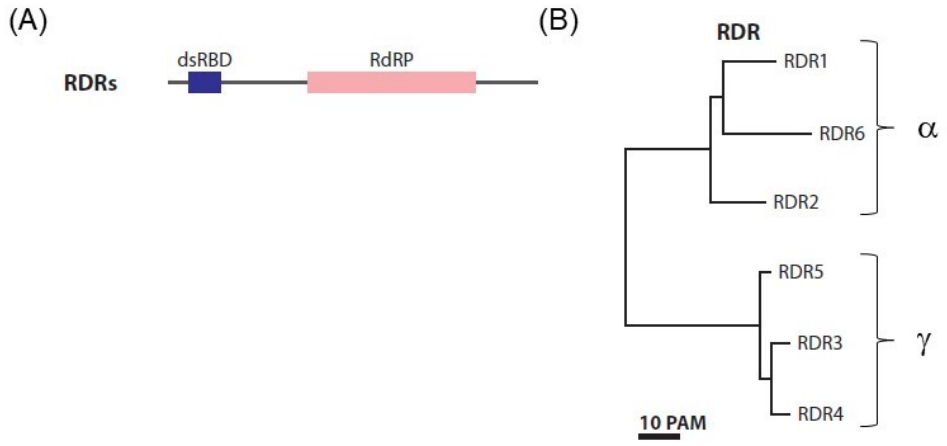


Figure 1.7 Les RDRs végétales

(A) Domaines fonctionnels des RDRs (Tiré de Bologna and Voinnet, 2014). (B) Arbre phylogénétique des RDRs (Modifié de Bologna and Voinnet, 2014).

1.4.4 Inhibition de l'interférence à l'ARN par les virus

Cohérent avec la nature antivirale de l'interférence à l'ARN, la majorité des virus ont développé des protéines effectrices, les *VIRAL SUPPRESSOR OF RNA SILENCING* (VSR), afin de neutraliser l'interférence à l'ARN (Voinnet et al., 1999). Les VSRs peuvent interférer avec pratiquement toutes les étapes de la voie de l'ARN interférence et permettent donc d'atténuer ou supprimer complètement la réponse de défense (Carbonell et Carington, 2015) (Figure 1.8). Par exemple, une stratégie de répression fréquente utilisée par plusieurs VSRs codés par divers genres de virus est l'expression de protéines pouvant séquestrer les siRNAs (P19, Hc-Pro, P21, P15, P130/P126/P122, γB, NS3, Pns10, NSs, etc.) (Csorba et al., 2015). Plusieurs de ces VSRs ont la capacité d'interagir avec les protéines AGO, via leurs motifs GW, ce qui mène souvent, mais pas toujours, à la dégradation des AGO via le protéasome. C'est le cas de la protéine P38 de TCV, la P0 du BWYV (Beet western yellow virus). D'autres parts, certains virus peuvent aussi indirectement inhiber l'ARN interférence; par exemple, en induisant l'expression de régulateurs négatifs de l'ARN interférence; par exemple l'induction de RTL1 par différents virus (Shamandi et al., 2015).

On a d'abord pensé que PVX ne codait pas pour un VSR (Voinnet et al., 1999). Néanmoins, des études subséquentes ont par la suite démontré que la P25 de PVX était en effet un VSR qui empêchait le mouvement du signal d'extinction en dehors de la cellule initialement infectée (2000). Les auteurs ont suggéré que la P25 pouvait interférer avec la production du signal mobile produit par le mécanisme de l'ARN interférence i.e. à l'étape de l'amplification du signal par les ARN polymérases ARN-dépendante de l'hôte (Voinnet et al., 2000). Plus récemment, des essais de co-immunoprécipitation ont montré que la P25 de PVX interagit avec AGO1, AGO2, AGO3 et AGO4 et qu'elle induit la dégradation d'AGO1 via la voie du protéasome (Chiu et al., 2010). Chez la plupart des membres de la famille des Potexvirus, à l'exception de PepMV dont la CP agit aussi comme un VSR, TGB1 est le VSR. De façon surprenante, ces VSRs encodés par les membres du même genre ont une efficacité très variable et agissent de façons différentes pour interférer avec la défense médiée par le mécanisme de l'ARN interférence (Csorba et al., 2015; Senshu et al., 2009). Par exemple, TGB1 de PlAMV

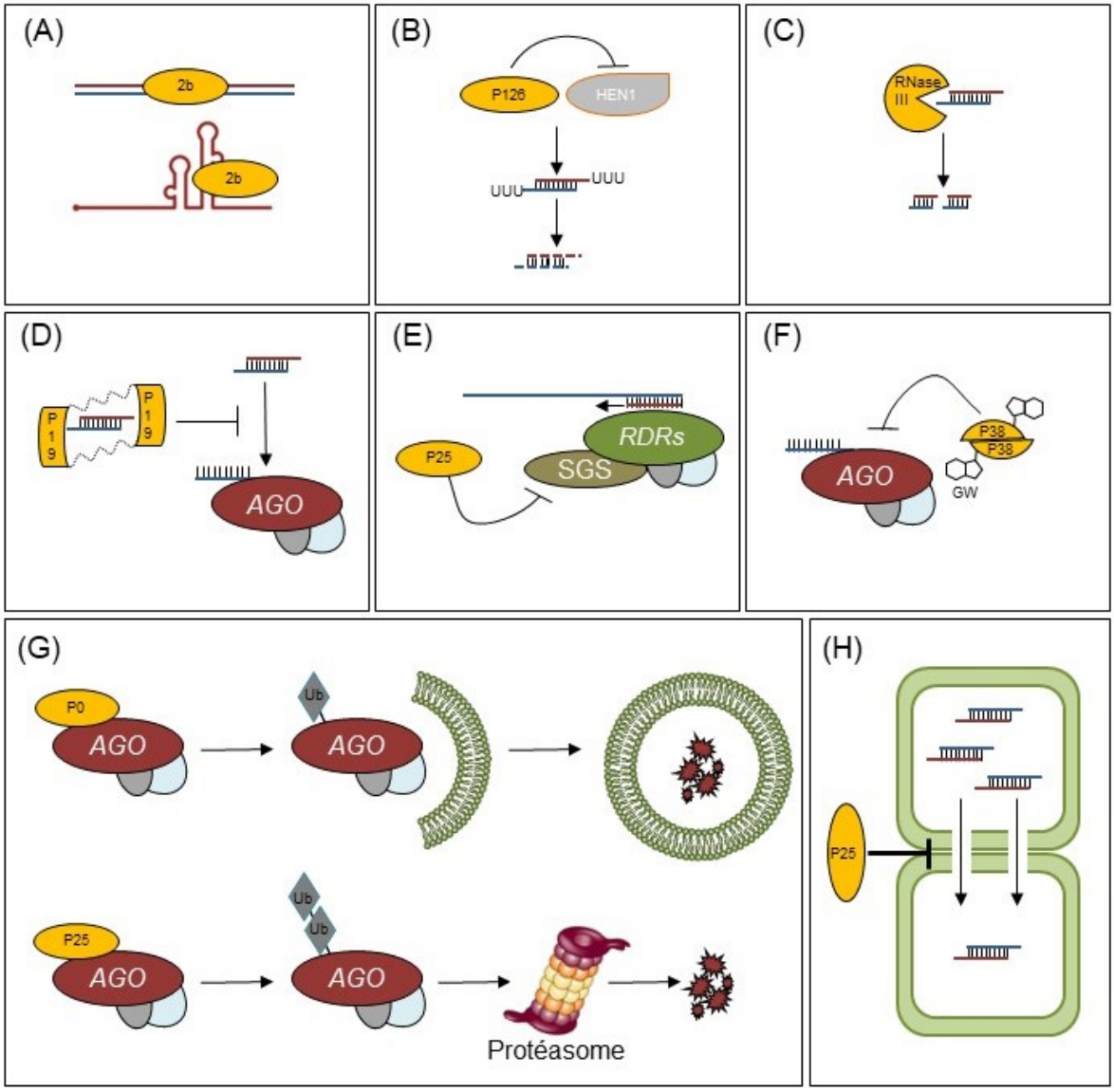


Figure 1.8 Schématisation des différents modes d'action des VSRs.

(A) En séquestrant les long précurseurs d'ARNdb, la protéine 2b du Cucumber mosaic virus (CMV) empêche l'action des DCL et ainsi, empêche la production des vsiARNs. (B) La P126 du Tobacco mosaic virus (TMV) prévient la méthylation des siARNs par HEN1 ce qui induit leur uridylation et leur dégradation. (C) L'endoribonucléase III (RNase III) du Sweet potato chlorotic stunt virus (SPCSV) degrade les sARNs en sARNs de 14-nt, qui eux sont inactifs. (D) La P19 des Tombusvirus lie et séquestre les sARNs de 21-nt ce qui prévient leur incorporation dans une protéine AGO. (E) La P25 de Plantago asiatica mosaic virus (PlAMV) altère la

localisation de SGS3, un co-facteur important pour l'amplification du signal (F) La P38 de TCV possède des motifs GW lui permettant de lier et inhiber AGO1. (G) La P0 des Poleovirus et la P25 des Potexvirus induit l'ubiquitination de certaines AGOs induisant ainsi leur dégradation via l'autophagie et le protéasome, respectivement. (H) La P25 du Potato virus X (PVX) permettrait d'interférer avec le mouvement des sARNs.

inhibe la synthèse d'ARNdb dépendente de RDR6/SGS3 en interagissant avec ces dernières dans des granules cytoplasmiques (Okano et al., 2014). La différence d'efficacité des VSRs de la famille des Potexvirus réflète grandement leur capacité à établir une infection systémique. Dans les dernières années, il a été démontré que TGB1 de PVX réorganise l'actine et les endomembranes dans les *X-bodies*, une usine de réplication virale (VRC) (Tilsner et al., 2012). Bien qu'aucune expérience n'ait pu vérifier cette hypothèse, il est proposé depuis quelques années que la formation de ces VRCs pourrait aussi agir en tant que VSR en empêchant la machinerie de l'hôte, tel que des nucléases, d'accéder aux acides nucléiques viraux. Il est intéressant de noter que plusieurs genres de virus (Carlavirus, Allexivirus, Foveavirus, Hordeivirus, Benyvirus, Pomovirus and Pecluvirus) codent pour TGB1 mais que ces protéines n'ont pas la capacité d'interférer avec le mécanisme de l'ARN interférence (Senshu et al., 2009). Ainsi, les protéines homologues codées par des membres de genres différents ne sont pas nécessairement des VSRs.

1.5 Réplication du Potato virus X (PVX)

PVX fait partie de la famille des *Alphaflexiviridae*, des virus possédant un génome à ARNsb de polarité positive (+) ayant une coiffe et une queue poly-A. Son génome encode cinq protéines soit, une ARN polymérase ARN dépendante (vRDR/hélicase), trois protéines de mouvement (P25/TGB1, 8K/TGB2; 12K/TGB3) et une protéine de capside (CP). La première étape du cycle d'infection est la décapsidation du génome i.e. la libération de l'ARN (+) dans le cytoplasme de la cellule hôte. En utilisant la machinerie traductionnelle de l'hôte, la vRDR sera produite. L'activité de cette dernière, qui se sert des membranes de l'hôte, mènera à la production du brin (-) qui sera suivie d'une succession de réplication du brin (+) et de la synthèse des ARN

subgénomiques (sgARN). Les sgARNs sont essentiellement des sections plus courtes du brin (+) de l'ARN viral transcrites à partir de promoteurs subgénomiques internes. La traduction de ces ARNg produit les protéines TGB1, TGB2, TGB3 ainsi que la protéine de capside.

Les protéines TGB, formant le *triple gene block*, ont un rôle crucial dans la réplication et le mouvement de PVX car elles induisent la formation des VRC qui contiennent plusieurs facteurs de l'hôte, les brins (-) et (+) de l'ARNv. TGB1 est une hélicase d'ARN qui fonctionne également comme un activateur de la traduction (Atabekov et al., 2000; Rodionova et al., 2003). TGB1 (ou P25) est le VSR de PVX. Certaines études ont démontré que TGB1 possède la capacité d'augmenter la limite d'exclusion des plasmodesmes et qu'elle interagit avec la CP et l'extrémité 5' du vARN pour former des complexes ribonucleoprotéiques permettant ainsi le mouvement de cellule-en-cellule (Atabekov et al., 2000; Rodionova et al., 2003). De plus, Tilsner et coll. ont récemment démontré que TGB1 co-localise avec la CP à l'intérieur des plasmodesmes suggérant que TGB1 pourrait aussi être nécessaire pour l'insertion de virions ou de complexes RNP dans les plasmodesmes (Tilsner et al., 2013). Finalement, de par sa capacité à réorganiser les filaments d'actine et les endomembranes, TGB1 est la protéine centrale pour la formation des complexes de réplication viral (VRC) ou X-bodies qui sont associés avec le ER de la cellule hôte. TGB1 recrute aussi deux protéines transmembranaires virales dans les VRCs soit, TGB2 et TGB3 (Tilsner et al., 2012).

Au cours des étapes initiales de l'infection par PVX, TGB2 induit la formation de granules comprenant TGB2/TGB3 localisés au réticulum endoplasmique (Krishnamurthy et al., 2003). Ces granules contiennent aussi la réplicase, des ribosomes et co-localisent avec les filaments d'actine suggérant que ces granules seraient un lieu de réplication et seraient mobiles. TGB3 serait le facteur permettant le mouvement de ces granules du ER perinucléaire vers le ER cortical (Solovyev et al., 2000; Schepetilnikov et al., 2005; Lee et al., 2010; Wu et al., 2011). Aux plasmodesmes, le complexe vRNP sera ancré grâce à TGB2/TGB3 (Park et al., 2014). À l'intérieur des plasmodesmes, le desmotubule est une structure membranaire tubulaire étroite et incurvée permettant la continuité entre les RE de deux cellules adjacentes. Certaines protéines spécifiques du ER cortical et des desmotubules, telles que les réticulons, permettent de maintenir

la courbure des tubules et seraient importantes pour la localisation de TGB3 dans les plasmodesme (Tilsner et al., 2011).

Au cours des étapes intermédiaires de l'infection par PVX, de plus en plus d'ARN viral et de protéines virales s'accumulent dans ces complexes de réplications ancrés aux plasmodesmes alors que les VRCs cytoplasmiques qui n'étaient pas ancrés au ER deviennent des granules mobiles liés aux membranes (Tilsner et al., 2011). Les 107 nucléotides en 5' du génome de PVX sont requis pour le mouvement (Lough et al., 2006) et contiennent une structure de tige boucle impliquée dans l'encapsidation du génome (Kwon et al., 2005; Verchot-Lubicz et al., 2007). Certaines études suggèrent qu'il pourrait y avoir encapsidation du génome dans les VRCs localisés aux plasmodesmes. TGB1 se lie spécifiquement à l'extrémité 5' des virions partiellement ou totalement encapsidés (Karpova et al., 2006). En interagissant avec la CP et le plasmodesme, TGB1 permet de déplacer l'ARN viral à l'intérieur du pore permettant ainsi le mouvement dans la cellule adjacente (Lough et al., 2006). Puisque TGB2 interagit avec l'ARNv, la CP et TGB1, il est possible que TGB2 puisse aussi être impliquée dans le transfert du virus dans le plasmodesme vers la cellule voisine.

Au cours des étapes tardives de l'infection par PVX, les granules TGB2/TGB3 s'accumule dans le VRC périnucléaire, le X-body, dans lequel TGB1 est l'organisateur central permettant de recruter les filaments d'actine et d'autres facteurs de l'hôte (Tilsner et al., 2013).

En plus d'être les principaux centres de réplication virale, les VRCs peuvent également faciliter l'accès des virus aux ressources essentielles de l'hôte, telles que les ribosomes, les enzymes et les nucléotides (Linnik et al., 2013).

1.6 Travaux antérieurs au projet de doctorat

Avant le début de mon projet de doctorat, très peu d'études avaient étudié l'implication de l'ARN interférence dans la défense contre PVX. En 2011, une étude de notre laboratoire avait permis d'identifier l'implication d'AGO2 dans la défense antivirale contre PVX chez

Arabidopsis (Jaubert et al., 2011). *Arabidopsis thaliana* Col-0 est résistante à PVX. Pour vérifier si l'ARN interférence était impliquée dans la résistance contre ce virus, des triple mutant DCL *dcl2 dcl3 dcl4* ainsi que des simples mutants pour les 10 AGOs d'*Arabidopsis* ont été inoculés avec PVX pour vérifier leur susceptibilité. Ils ont noté que PVX était capable d'établir une infection systémique dans le mutant *dcl2 dcl3 dcl4* suggérant que l'ARN interférence est bel et bien responsable du phénotype de résistance observé chez *A. thaliana.* Parmi tous les mutants AGOs testés, seul le mutant *ago2* montrait le mouvement systémique de PVX et une accumulation de PVX dans les feuilles non-inoculées mais à un niveau moindre que celui observé chez le mutant *dcl2 dcl3 dcl4* suggérant (1) qu'AGO2 est importante pour la résistance contre PVX chez *Arabidopsis* (2) qu'une ou plusieurs autres protéines sont impliquées dans le phénotype de résistance. Pour cette raison, nous avions l'hypothèse qu'une ou plusieurs AGOs assiste AGO2 dans la défense contre PVX. Cette étude de 2011 était très innovatrice puisque la plupart des interactions plante-virus avaient été étudiées dans un contexte où le virus en question est capable de surmonter l'ARN interférence. Le système PVX-*Arabidopsis* était l'un des seuls exemples où l'ARN interférence permettait d'empêcher l'infection systémique par un virus sauvage i.e. codant pour son VSR. L'utilisation de virus de type sauvage pour étudier le mécanisme de l'ARN interférence est importante puisque, dans de nombreux cas, les VSRs sont des protéines multifonctionnelles. Par exemple, la protéine PVX P25 est essentielle pour le mouvement du virus ainsi que pour la formation des *X-bodies* associés à la réplication, ces derniers pouvant probablement protéger physiquement les génomes viraux des mécanismes de défense hôte (Tilsner et al., 2012). Cependant, le rôle des structures subcellulaires induites par le virus dans la défense contre l'ARN interférence n'a pas été étudié. Il est fort à parier que d'autres protéines AGO ou peut-être même toutes les AGO ont probablement des fonctions se chevauchant et / ou épistatiques concernant la défense antivirale. Le type d'infection virale (par exemple la localisation ou la formation de structures intracellulaires spécifiques) et la nature des suppresseurs pourraient également déterminer quels AGO sont recrutés le plus efficacement par la plante pour une fonction de défense antivirale dans les conditions existantes.

Arabidopsis encode 10 protéines AGO chacune étant probablement spécialisées dans différentes voies de l'ARN interférence, dont certaines ont été montrées pour être impliquées dans la

défense antivirale (AGO1, AGO2, AGO7). Parmi celles-ci, il semble qu'AGO2 joue un rôle central dans la défense puisqu'elle a été montrée pour être impliquée dans la défense contre un large éventail de virus, y compris CMV, TCV, TRV, PVX, TuMV (Harvey et al., 2011; Wang et al., 2011; Zhang et al., 2012; Ma et al., 2015; Jaubert et al., 2011; Carbonell et al., 2012). Tel que mentionné précédemment, PVX WT n'infecte pas systémiquement *Arabidopsis* Col-0 mais est capable d'infecter un mutant *ago2* suggérant qu'AGO2 pourrait, en partie, déterminer la gamme d'hôte. De façon intéressante, une étude de notre laboratoire a démontré que la fonction antivirale d'AGO2 est conservée chez *Nicotiana benthamiana* (Scholthof et al., 2011). Cette étude démontrait que les plants dont l'expression d'AGO2 était compromise (par VIGS) permettaient une accumulation plus importante du Tomato bushy stunt virus delta P19 (TBSVΔP19)(Scholthof et al., 2011). Malgré la présence d'une AGO2 antivirale, *N. benthamiana* est fortement susceptible à de nombreux virus incluant PVX suggérant que les différences observées au niveau de la séquence codante d'AGO2 de *A. thaliana* et *N. benthamiana* contribuent au phénotype de résistance ou susceptibilité face à PVX observé chez ces deux espèces respectivement. Bien que différentes études aient identifié les acides aminés fonctionnels dans les protéines AGO importants pour leurs fonctions essentielles (Poulsen et al., 2013; Fatyol et al., 2016), peu de recherches ont été menées sur la manière dont les différences entre les protéines AGO pouvaient affecter l'infection virale. Quelles sont les bases expliquant la spécialisation fonctionnelle antivirale de certaines AGOs?

1.7 Objectifs du projet de recherche

Plusieurs espèces végétales de la famille des Solanacées démontrent une grande susceptibilité face aux virus du genre Potexvirus (Mathioudakis et al., 2013) malgré la présence du mécanisme de l'ARN interférence. Tel que discuté précédemment, chez la plante modèle *Arabidopsis thaliana* Col-0, il semble que l'ARN interférence suffit à rendre la plante immune à PVX. Nous émettons donc l'hypothèse que la manipulation ou l'ajout de gènes impliqués dans le mécanisme de l'ARN interférence chez les espèces susceptibles au Potexvirus pourrait permettre de conférer une résistance accrue.

Pour ce faire, le premier objectif était d'identifier et de caractériser quelles sont les protéines AGO ayant une activité antivirale contre les Potexvirus et de vérifier l'impact de la formation des *X-bodies* associés à la réplication et de la présence du VSR sur l'activité de ces protéines. Pour ces travaux, nous avons utilisés deux modèles viraux soit : dans un premier temps, PVX, un virus incapable de surmonter l'ARN interférence chez *Arabidopsis* et PlAMV, un virus capable d'établir efficacement une infection systémique chez *Arabidopsis*. Dans ce projet, nous cherchions d'abord à savoir s'il existe des AGOs spécifiques à un genre de virus i.e. de vérifier si les protéines AGOs identifiés comme étant antivirales contre PVX, le sont aussi contre un autre Potexvirus, PlAMV. Notre hypothèse de départ était que PlAMV est capable d'infecter *Arabidopsis* parce qu'il possède la capacité de compromettre l'activité de toutes les protéines AGOs antivirales. De plus, si seulement un sous-groupe des protéines AGO se démontre être antivirales contre les Potexvirus, la comparaison de ces AGOs entre-elles pourrait permettre de comprendre quelles sont les bases expliquant la spécialisation fonctionnelle antivirale de certaines AGO. Selon nos résultats obtenus, cet objectif pourrait aussi nous aider à comprendre quels sont les signaux moléculaires qui dictent les activités antivirales coordonnées et coopératives des AGOs dans les différents stades de développement et tissus des plantes.

Le second objectif était de caractériser comment la variabilité retrouvée dans la séquence codante d'AGO2 module l'activité antivirale de la protéine face à PVX. En d'autres mots, au niveau moléculaire, un travail plus structurel était nécessaire pour mieux comprendre ce qui permet une AGO d'être antivirale contre les Potexvirus.

CHAPITRE 2

IDENTIFICATION DU RÔLE D'ARABIDOSPSIS AGO5 DANS LA DÉFENSE ANTIVIRALE PAR ANALYSE FONCTIONNELLE ET GÉNÉTIQUE

Dans cet article, nous avons démontré, à l'aide d'essais fonctionnels et génétiques, que la protéine AGO5 d'*Arabidopsis* contribue, conjointement avec AGO2, à l'immunité antivirale de cette plante contre PVX. Ces résultats sont d'une grande importance pour la recherche dans ce domaine, puisqu'il s'agit de la toute première preuve de l'implication d'AGO5, une protéine exclusivement exprimée dans les tissus reproductifs en conditions de croissance normales, dans la défense antivirale. De façon encore plus importante, nous démontrons que toutes les protéines AGO d'*Arabidopsis* possèdent la capacité intrinsèque de reconnaître et cibler l'ARN viral lorsque cet ARN n'est ni protégé par l'action d'un VSR ou par un complexe de réplication viral intact. Ces résultats vont donc à l'encontre de l'idée préconçue dans ce domaine d'une spécialisation des protéines AGO où il était présumé que seulement certaines protéines AGO avaient cette capacité antivirale.

Pour cet article, CB et PM ont conçu les expériences conjointement. CB a réalisé toutes les expériences. La rédaction du manuscrit a été faite par CB et PM. Cet article a été publié dans la revue scientifique *The plant cell* : Brosseau, C. and Moffett, P. (2015). Functional and Genetic analysis Identify a Role for *Arabidopsis* ARGONAUTE5 in Antiviral RNA Silencing. Plant Cell *27*, 1742-1754.

2. FUNCTIONAL AND GENETIC ANALYSIS IDENTIFY A ROLE FOR ARABIDOPSIS ARGONAUTE5 IN ANTIVIRAL RNA SILENCING

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2.1 Abstract

RNA silencing functions as an anti-viral defence through the action of DICER-like (DCL) and ARGONAUTE (AGO) proteins. In turn, plant viruses have evolved strategies to counteract this defence mechanism, including the expression of suppressors of RNA silencing. *Potato virus X* (PVX) does not systemically infect *Arabidopsis* Col-0, but is able to do so effectively in mutants lacking at least two of the four *Arabidopsis* DCL proteins. PVX can also infect *Arabidopsis ago2* mutants, albeit less effectively than double DCL mutants, suggesting that additional AGO proteins may mediate anti-viral defenses. Here we show, using functional assays, that all *Arabidopsis* AGO proteins have the potential to target PVX lacking its VSR, P25, but that only AGO2 and AGO5 are able to target wild type PVX. However, P25 directly affects only a small subset of AGO proteins, and we present evidence indicating that its protective effect is mediated by precluding AGO proteins from accessing viral RNA, as well as by directly inhibiting the RNA silencing machinery. In agreement with functional assays, we show that Potexvirus infection induces *AGO5* expression and that both AGO2 and AGO5 are required for full restriction of PVX infection in systemic tissues of *Arabidopsis*.

2.2 Introduction

Plants employ multiple defence mechanisms to restrict virus replication and movement (Incarbone and Dunoyer, 2013). RNA silencing is employed by plants to counteract invading nucleic acids, including viruses and is conserved in most eukaryotic organisms (Ding and Voinnet, 2007). RNA silencing refers collectively to diverse RNA-based processes that are triggered by the presence of double stranded RNA (dsRNA). In the case of defense against single stranded RNA viruses (ssRNA), dsRNA arises from replication intermediates as well as highly structured ssRNA. This viral dsRNA is recognized and cleaved into small interfering RNAs (siRNAs), or viral small interfering RNAs (vsiRNAs), by Dicer-like (DCL) proteins. After incorporation of these vsiRNAs duplexes into an RNA-induced silencing complex (RISC), one strand of the duplex is then used as a sequence-specific guide to suppress gene expression of complementarity ssRNA either by cleavage or translational repression (Voinnet et al., 2009;

Baulcombe, 2004). Although the exact composition of RISC complexes is still unclear, the core components of these complexes are the RNase H-like Argonaute (AGO) proteins (Iwakawa and Tomari, 2013).

Plant viruses are able to counteract RNA silencing defense mechanisms by expressing viral suppressors of RNA silencing (VSRs). VSRs have been identified in almost all plant virus genera and are highly diverse. VSRs may affect different steps of RNA silencing by sequestering sRNAs or by inhibiting RISC assembly, sRNA methylation, or silencing signal amplification (Pumplin and Voinnet, 2013). In addition, multiple VSR proteins have been shown to directly target AGO proteins, resulting in an inhibition of RISC formation, or by destabilization of the AGO protein (Csorba et al., 2015). The triple gene block protein 1 (TGB1) of potato virus X (PVX), also known as P25, suppresses movement of a systemic silencing signal (Voinnet et al., 1999; Voinnet et al., 2000).

Arabidopsis encodes ten Argonaute (AGO) proteins that are thought to be specialized to function in different RNA silencing-related mechanisms (Mallory and Vaucheret, 2010), some of which have been implicated in antiviral defense. Hypomorphic *ago1* mutants have been shown to be more susceptible to *Brome mosaic virus* (BMV), *Cucumber mosaic virus* (CMV) and to VSR-defective CMV and *Turnip crinkle virus* (TCV) (Dzianott et al., 2012; Morel et al., 2002; Qu et al., 2008; Azevedo et al., 2010). However, an *ago1* mutant has been shown to be less susceptible to *Tobacco rattle virus* (TRV), while AGO1 was found to be required for virus-induced gene silencing (Ma et al., 2015). In addition, an *ago7* mutant has been shown to be more susceptible to specific derivatives of VSR-defective TCV (Qu et al., 2008) and *ago4* mutants are more susceptible to TRV (Ma et al., 2015). In contrast, AGO2 appears to be broadly required for anti-viral defenses, having been shown to be involved in defense against a wide range of viruses, including CMV, TCV, TRV, *Potato virus X* (PVX), *Turnip mosaic virus* (TuMV), and *Tomato bushy stunt virus* (TBSV) (Harvey et al., 2011; Wang et al., 2011; Zhang et al., 2012; Ma et al., 2015; Jaubert et al., 2011; Carbonell et al., 2012; Scholthof et al., 2011). It has also been shown that RISC complexes containing AGO1, 2, 3 or 5 act on viruses in an *in vitro* Tombuvirus replication assay and that AGO1, 2, 3, 4, 5, and 9 can all bind to sRNAs derived

from viruses or viroids (Takeda et al., 2008, Wang et al., 2011; Minoia et al., 2014; Schuck et al., 2013). These observations suggest that multiple AGO proteins may have the intrinsic ability to bind vsiRNA and target viral RNAs, but raise the questions of which AGO proteins have antiviral functions in a biological context and whether different AGO proteins may have cooperative or redundant functions.

PVX is the type member of the Potexvirus family of viruses (Verchot-Lubicz et al., 2007). Although the PVX VSR, P25 destabilizes *Arabidopsis* AGO1, this virus does not normally systemically infect *Arabidopsis* Col-0 (Chiu et al., 2010; Jaubert et al., 2011). However, PVX can effectively infect *Arabidopsis* if a VSR is supplied *in trans* from a second virus or if RNA silencing is attenuated by mutation of DCL-encoding genes or *AGO2* (Jaubert et al., 2011; Bagus Andika et al., 2015). At the same time, PVX accumulates to a lesser extent in the *ago2* mutant than it does in a *triple dicer* (*dcl2*/*dcl3*/*dcl4*) mutant (Jaubert et al., 2011), suggesting that at least one other AGO protein may function in resistance to PVX in *Arabidopsis* in concert with AGO2. The PVX/*Arabidopsis* system is one of the only examples where infection by a wild-type virus has been shown to be dramatically restricted due to an inability of its VSR to overcome the RNA silencing mechanisms of a specific host. The use of wild-type viruses to study RNA silencing may be important in many cases as VSRs are often multifunctional proteins. For example, the PVX P25 protein has been shown to be essential for cell-to-cell movement, as well as for the formation of replication-associated X-bodies, the latter which have may physically protect viral genomes from host RNA silencing machinery (Tilsner et al., 2012). However, the role of virus-induced subcellular structures in defense against RNA silencing has not been investigated.

We have systematically investigated the involvement of different AGO proteins in defence against PVX in *Arabidopsis* using functional and genetic analysis. In a functional assay based on transient expression in *Nicotiana benthamiana*, we find that *Arabidopsis* AGO1, 2, 3, 4, 5, 6, 7, 9 and 10 all have the ability to target PVX, but only if the latter lacks its VSR, P25. In contrast, only *Arabidopsis* AGO2 and AGO5 have the ability to target WT PVX in *N. benthamiana*. This dichotomy is not strictly explained by a direct action of P25 on AGO proteins

as P25 compromised the accumulation of only AGO1 and AGO7. This suggests that P25 may protect PVX by precluding access to viral RNA by virtue its role in the formation of X-bodies, in addition to targeting RNA silencing mechanisms directly. We suggest that AGO2 and AGO5 function in antiviral defense due to their ability to bind and inhibit viral RNA despite the formation of such structures. In agreement with our functional analysis, genetic analysis demonstrated a novel role for AGO5 in anti-viral defense, indicating that this protein functions in cooperation with AGO2. Indeed, a double *ago2*/*ago5* mutant showed similar susceptibility as a triple dicer mutant, indicating that these two proteins are the major AGO proteins responsible for RNA silencing against PVX in *Arabidopsis*. However, this cooperation appears to have a temporal/spatial aspect in that AGO5 was found to be important in curtailing systemic PVX infection only once AGO2 is overcome in initially infected leaves.

2.3 Results

2.3.1 *Arabidopsis* AGO2 and AGO5 act synergistically to compromise PVX accumulation in *N. benthamiana*

As no other single *ago* mutant allows for PVX accumulation in *Arabidopsis*, we undertook a functional approach by transiently overexpressing *Arabidopsis* AGO proteins together with PVX-GFP in *N. benthamiana* leaves. This included two versions of AGO1 (1S and 1L, which differ by a two amino acid deletion in AGO1S), AGO2 to 7, AGO9 and AGO10, but not AGO8 as it is thought to be a pseudogene (Takeda et al., 2008). Consistent with the genetic analyses, transient expression of AGO2 resulted in a lower accumulation of PVX-derived GFP, as determined visually and by immune-blotting (Figure 1A and 1B). Although all AGO proteins tested were expressed, only AGO5 caused a similar effect (Figure 1A and 1B). Co-expression of both AGO2 and AGO5 induced an even greater decrease in virus-derived GFP (Figure 1C and 1D; Supplemental Figure 1C and 1D).

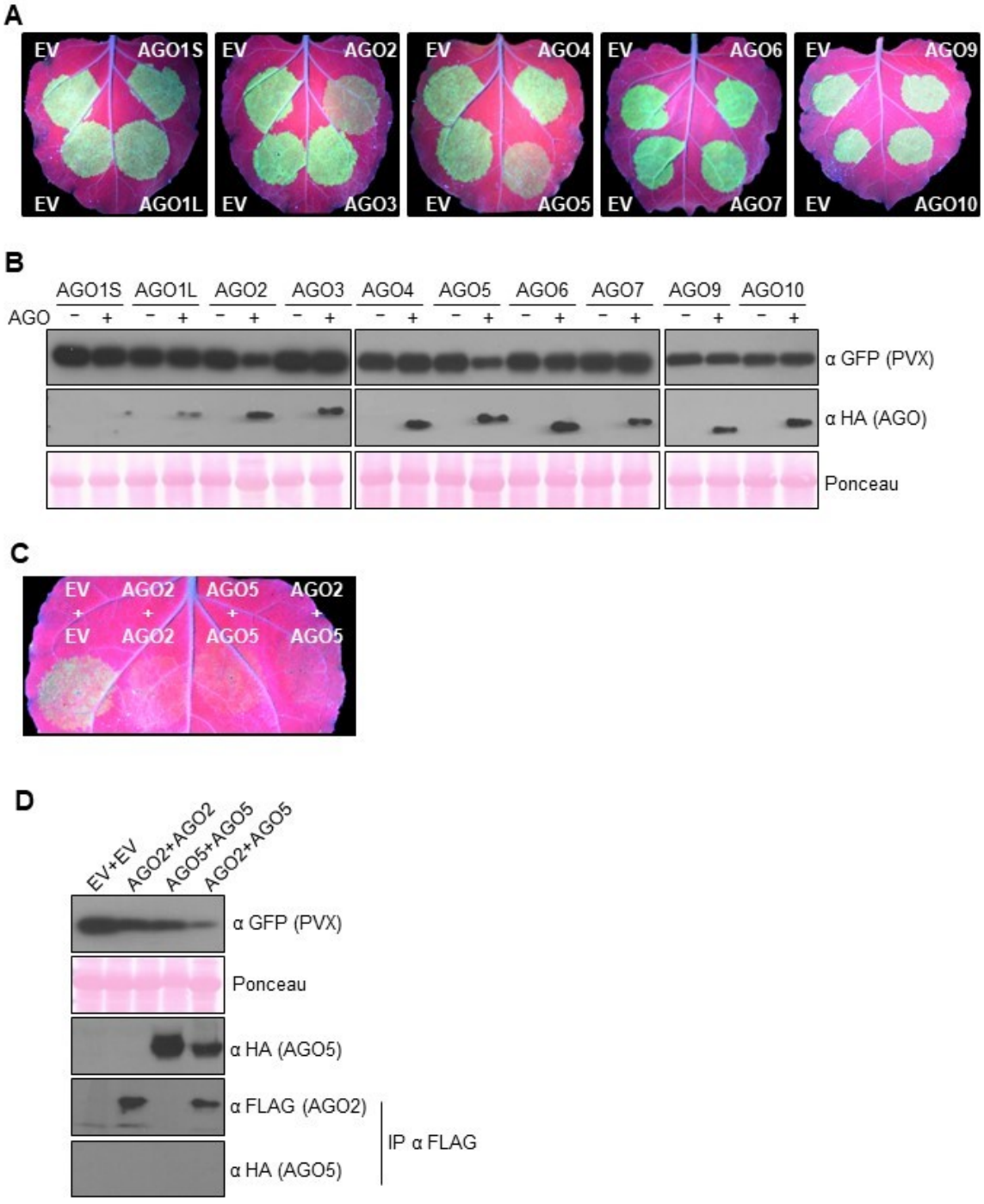

Figure 1. AGO2 and AGO5 act synergistically to counteract PVX accumulation in *N. benthamiana.* (A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFP along with ten different HA-tagged Arabidopsis AGO proteins or empty vector (EV), as indicated. Leaves were

photographed under UV illumination 4 days post infiltration (dpi). (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). (+) indicates that presence of the indicated AGO protein and (-) indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. (C) PVX-GFP was agroinfiltrated along with EV, FLAG-AGO2 or HA-AGO5 alone or in combination, as indicated. Leaves were photographed under UV illumination 4 dpi. (D) Total protein extracts prepared from leaves agroinfiltrated as in (D) and subjected to anti-GFP immune-blotting (top panel). Aliquots from the same extracts were also subjected to anti-HA and anti-FLAG immunoprecipitation (IP) followed by anti-FLAG and/or anti-HA and immune-blotting. Ponceau staining of the same extracts is shown to demonstrate equal loading. Representative pictures are shown of experiments performed eight times with at least three plants per treatment.

In a parallel set of experiments, we co-expressed AGO2 and/or AGO5 with PVX expressing luciferase (PVX-LUC) along with 35S:R-LUC for normalization. Quantification of luciferase assays confirmed that only AGO2 and AGO5 reduced luciferase activity significantly (40% and 35% respectively), with AGO1 inducing only a minor (6%) decrease (Supplemental Figure 1A and 1B). Furthermore, AGO2 and AGO5 had an additive effect in reducing accumulation of virally-encoded protein (Supplemental Figure 1C and 1D). Although co-immunoprecipitation experiments did not detect physical interaction between AGO2 and AGO5, these results suggest that both AGO2 and AGO5 act non-redundantly to restrict PVX accumulation in *Arabidopsis*.

2.3.2 All *Arabidopsis* Argonaute proteins have the ability to target viral RNA

Several studies have shown that the presence of viral suppressor of RNA silencing (VSR) can modulate plant-virus interactions and the effectiveness of RNA silencing. For this reason, many studies on RNA silencing have used viruses lacking their VSR in order to identify which AGO protein is implicated in defense against viruses. To test this, we transiently expressed the same

ten *Arabidopsis* AGO proteins in *N. benthamiana* leaves with a version of PVX lacking the three triple gene block proteins (PVX-GFPΔTGB). Surprisingly, overexpression of all *Arabidopsis* AGO proteins tested compromised accumulation of PVX-GFPΔTGB-expressed GFP in *N. benthamiana*, as assessed visually and by immune-blotting, albeit to different degrees (Figure 2A and 2B). Similar results were seen with a PVX mutant lacking only P25 (Supplemental Figure 2) Importantly, none of the AGO proteins affected GFP accumulation expressed from a 35S:GFP construct (Supplemental Figure 3). These results suggest that all *Arabidopsis* AGO proteins can specifically recognize and target viral RNAs.

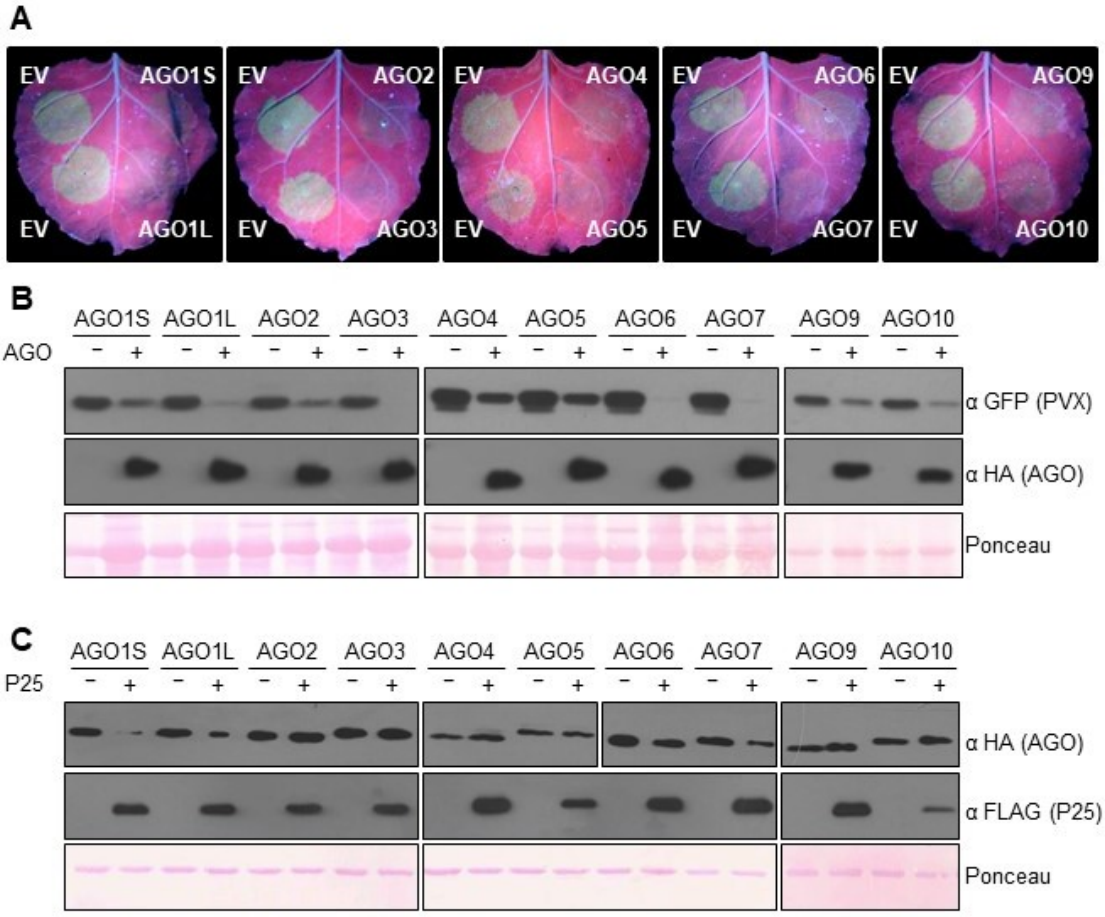

Figure 2. All *Arabidopsis* Argonautes can target viral RNA. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFPΔTGB along with either empty vector (EV) or HA-tagged *Arabidopsis* AGO proteins, as indicated. (A) Leaves were photographed under UV illumination 4 days post infiltration (dpi). Representative pictures are shown of experiments performed six

times with at least three plants per treatment. (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). (+) indicates that presence of the indicated AGO protein and (-) indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. (C) HA-tagged AGO proteins were coexpressed by agroinfiltration in *N. benthamiana* leaves with either FLAG-tagged P25 (+) or with empty vector (-). Total proteins were extracted and subjected to anti-FLAG immune-blotting. HA-tagged AGO proteins were immunoprecipitated and subjected to anti-HA immunoblotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Experiments have been performed three times with similar results.

2.3.3 Catalytic residues are required for effective PVX antiviral activity of AGO2 and AGO5 but dispensable for viral RNA binding.

AGO-mediated repression of target RNAs may occur through direct cleavage, destabilization or translational repression. However, some AGO proteins could have an indirect effect through association with endogenous miRNAs (Seo et al., 2013; Zhang et al., 2011). We therefore tested if the AGO2 and AGO5 proteins could bind PVX RNA. Because a previous study has demonstrated that catalytically inactive AGO proteins associate more stably with target RNAs, the second key residues of the catalytic triads of AGO2 and AGO5 were mutated to alanine (Carbonell et al., 2012). HA epitope tagged wild-type and catalytically dead mutants were co-expressed with PVX-LUC or PVX-GFP in *N. benthamiana* leaves. Three days after agroinfiltration, protein extracts were subjected to anti-HA immunoprecipitation. AGO/RNA interaction was assessed by extracting RNA from the immunoprecipitates, followed by RT-PCR using PVX-specific primers. Interestingly, both WT and mutant variants of AGO2 and AGO5 pulled down PVX RNAs with similar efficiency. In contrast, AGO9, which affects PVX-GFPΔTGB but not WT PVX (Figure 1A, Figure 2A), was found to associate only with the mutant version of PVX (Figure 3A). To confirm association between AGO proteins and PVX RNA, we inserted a streptavidin aptamer sequence (Srisawat and Engelke, 2001) into the PVX

genome (PVX-S1). This allowed us to pull down PVX RNA and verify the presence of specific proteins bound to viral RNAs by western blot analysis. No AGO proteins were detected in samples where we expressed PVX-GFP lacking the aptamer sequence (Supplemental Figure 5, second panel). However, upon pull down of PVX-S1 after co-expression with AGO2, AGO5 or AGO9, we detected AGO2 and AGO5, but not AGO9 in the pulled-down fraction (Supplemental Figure 5).

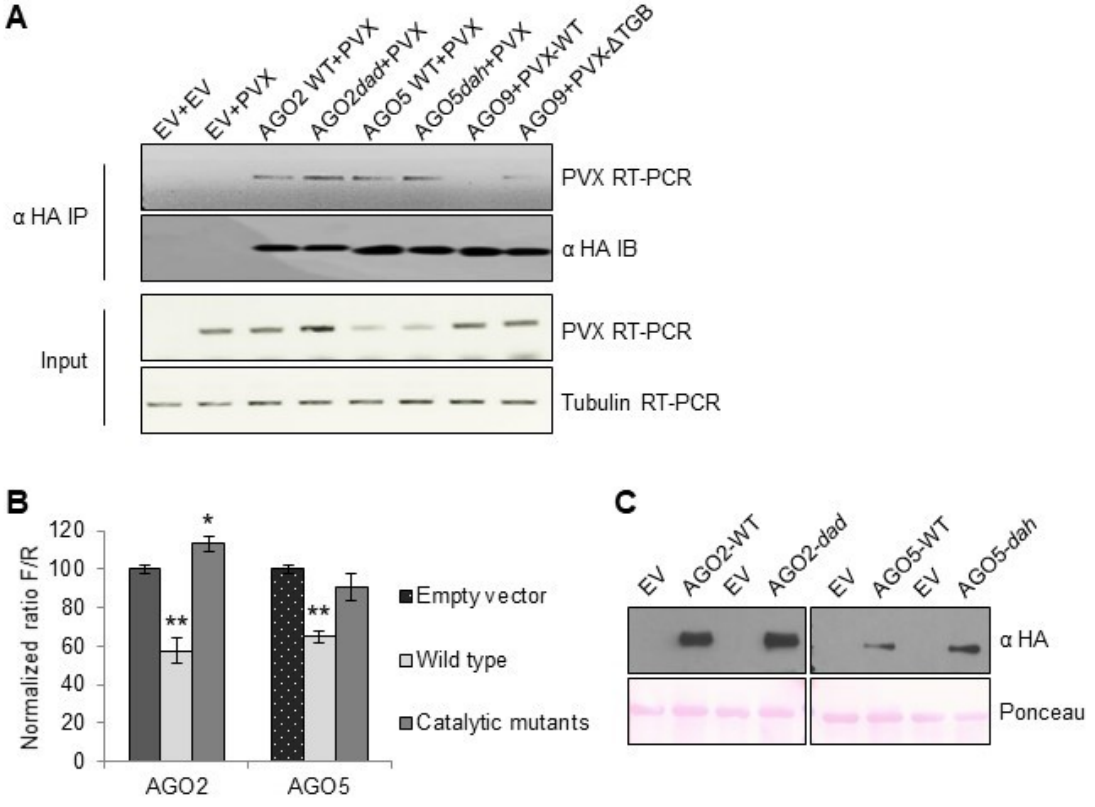

Figure 3. Catalytic activity of AGO2 and AGO5 is required for efficient defense against PVX, but is dispensable for viral RNA binding. (A) *N. benthamiana* leaves were agroinfiltrated with empty vector (EV) or with PVX-GFP together with either HA-tagged AGO2 or AGO5 or their catalytically dead mutant derivatives *dad* and *dah*, respectively, as indicated. HA-AGO proteins were immunoprecipitated (IP) from total protein extracts prepared 3 days post infiltration (dpi). RNAs from input and from HA immunoprecipitated fractions were extracted and submitted to RT-PCR analysis with PVX specific primers. Tubulin RT-PCR was

used as a control. Similar treatments were carried out by co-expressing HA-AGO9 with PVX-GFP or PVX-GFPΔTGB as indicated. This experiment was performed three times with similar results. (B) *N. benthamiana* leaves were agroinfiltrated with PVX-F-LUC and 35S:R-LUC together with either EV or WT or catalytically inactive AGO2 or AGO5. At 4 dpi firefly and renilla luciferase activities were measured from total protein extracts prepared from infiltrated tissues. Bar plots represents F-LUC activity normalized to R-LUC activity. Values represent the means ± SEM from three independent experiments (n=9). Data sets marked with * or ** are significantly different from empty vector infiltrated leaves as assessed by Student's t test at P-values < 0.05 and 0.0001, respectively. (C) HA-tagged AGO proteins were immunoprecipitated from total extracts from (B) and subjected to anti-HA immune-blotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

Because slicer-competent AGOs were found to associate with PVX RNAs, we hypothesized that these AGO might repress PVX-derived protein accumulation by translational repression wherein catalytic activity is dispensable. To investigate this possibility, we monitored luciferase activity from PVX-LUC in the presence of both variants of the AGO proteins. Mutation in the active site compromised the antiviral activity of both AGO2 and AGO5. Moreover, we observed in a significantly higher accumulation of luciferase when PVX-LUC was when co-expressed with a slicer-defective variant of AGO2, (Figure 3B and 3C). Similar results were seen with PVX-GFP (Supplemental Figure 6). These results suggest that the mutant AGO2 protein might act as a dominant negative by inhibiting endogenous AGO2, similar to previously described results with TuMV (Carbonell et al., 2012). Together, these results suggest that AGO2 and AGO5 directly target PVX RNA by cleavage. Given the fact that both WT and catalytically dead mutants bind PVX RNAs, it also suggests that AGO2 and AGO5 catalytic residues are not essential for passenger strand clearance from siRNA duplexes in this context.

2.3.4 Antiviral activity of AGO2 and AGO5 requires small RNAs

A number of reports have characterized the production of vsiRNAs upon infection by RNA viruses (Donaire et al., 2008; Takeda et al., 2008; Wang et al., 2011). These studies have shown

that the 21 nt vsiRNAs are the most abundant in infected plants, followed by 22-nt vsiRNAs. However, as previously noted in the case of TuMV, vsiRNA abundance is a poor indicator of antiviral silencing activity (Garcia-Ruiz et al., 2010). To investigate which class of vsiRNAs are required for the antiviral activities of AGO2 and AGO5 against PVX we performed overexpression assays in the presence of several VSRs. These included VSRs that sequester different sized siRNAs, including P14, (all sizes of siRNAs), P15, P21, and P19, (21 nt only) (Mérai et al., 2006), all of which were functional in a separate silencing suppression assay (Supplemental Figure 7). Co-expression of P14 with PVX-LUC completely compromised the antiviral activities of both AGO2 and AGO5. In contrast, the 21-nt-sequestering proteins P19, P15 and P21 partially attenuated the activity of AGO2 but completely inhibited that of AGO5 (Figure 4A). PVX-derived luciferase accumulation was reduced by approximately 15% in the presence of VSRs sequestering 21-nt sRNAs compared to a reduction around 35% without any VSR (Figure 4A). None of the VSRs affected the accumulation of AGO2 or AGO5 and co-expression of P14 resulted in inhibition loss of AGO2 and AGO5 association with PVX, as assessed by RNA immunoprecipitation (Figure 4B, 4C). These results suggest that optimal AGO2 antiviral activity is mediated through both 21 nt and longer small RNAs. In contrast, AGO5 activity is completely abolished by all VSRs tested, suggesting that AGO5 activity against PVX depends mainly on 21-nt sRNAs.

To further characterize the requirement of small RNAs for optimal antiviral defense response, we challenged single, double and triple *dcl* mutant plants with PVX and followed the accumulation of PVX in both local and systemic tissues at 5 and 21 dpi respectively. Mutation of both *dcl2* and *dcl4* allowed for PVX accumulation in inoculated leaves, although *dcl4* had a much greater effect than *dcl2* (Figure 5A). In systemic tissues, no significant difference in PVX accumulation was observed between these two single mutants, whereas the *dcl2*/*dcl4* double mutant allowed higher accumulation of PVX than either single mutant.

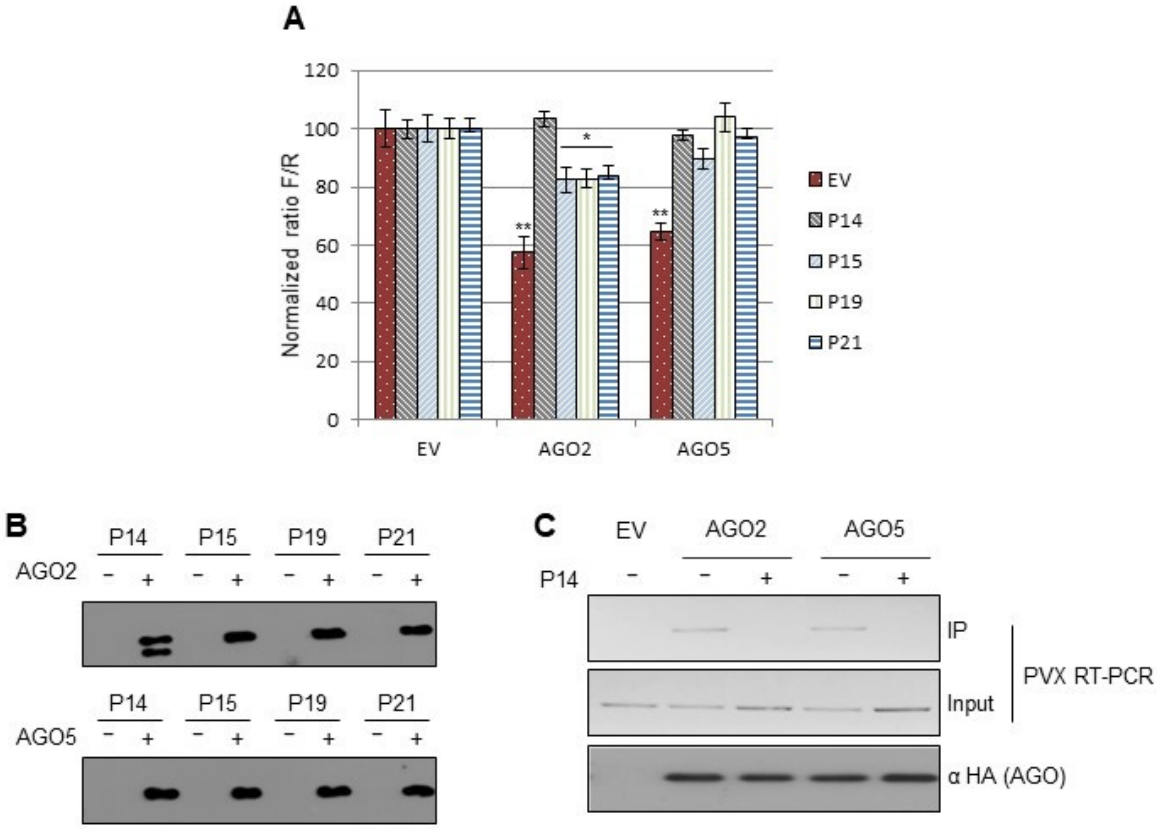

Figure 4. Antiviral activities of AGO2 and AGO5 depend mainly on 21-nucleotide small interfering RNAs. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-LUC and 35S:R-LUC, together with combinations of the VSRs, P14, P15, P19 and P21 as well as EV, AGO2 or AGO5, as indicated. Luciferase activities were measured from protein extracts prepared from infiltrated tissues 4 days post infiltration (dpi). Bar plots represent F-LUC activity normalized to R-LUC activity. Values represent means ± SEM from three independent experiments (n=9). Data sets marked with one or two asterisks are significantly different from empty vector infiltrated leaves as assessed by Student's t test at P-value < 0.05 or 0.005, respectively. (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) at 4 dpi. HA-tagged AGO 2 and AGO5 proteins were immunoprecipitated and subjected to SDS-PAGE followed by anti-HA immunoblotting. (C) *N. benthamiana* leaves were agroinfiltrated with PVX-GFP, together with combinations of P14 as well as EV, AGO2 or AGO5. At 4 dpi,

total proteins were extracted and subjected to immunoprecipitation (IP) with anti-HA antibodies. Subsequently, RNA from input and IP fractions were extracted and subjected to RT-PCR with PVX-specific primers. This experiment has been performed three times with similar results.

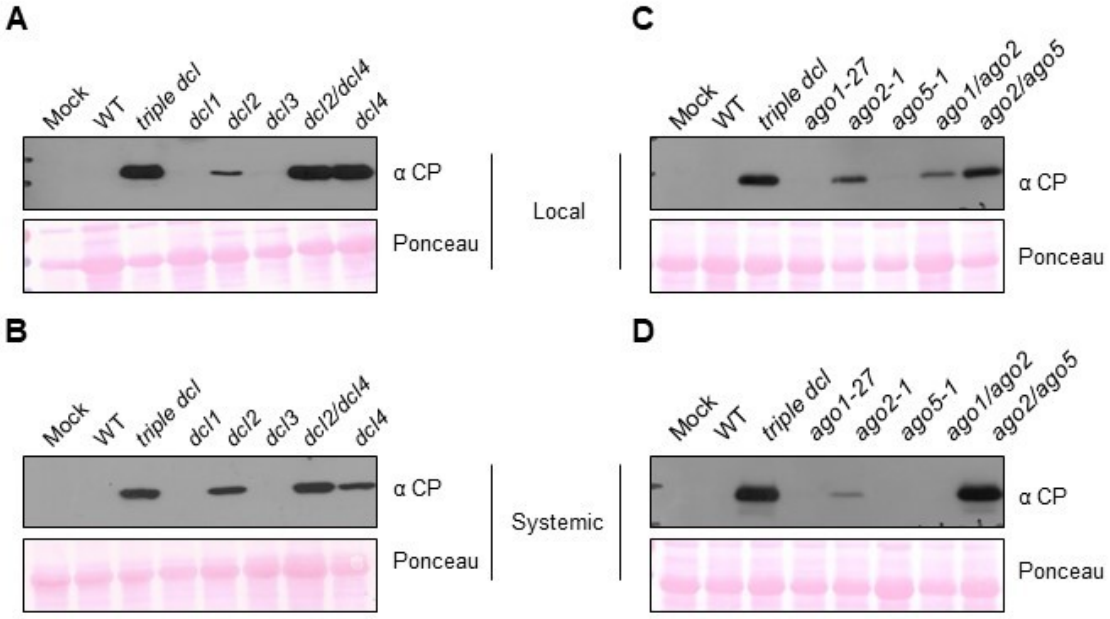

Figure 5. *Arabidopsis* mutants reveal additive effects of DCL2 and DCL4 and of AGO2 and AGO5 in systemic PVX infection. Col-0 WT *Arabidopsis* as well single, double or triple mutant *dcl* and *ago* mutant lines, as indicated, were infected with PVX. At 5 dpi (A, C) and 21 dpi (B, D) total protein extracts were prepared from inoculated and systemic leaves, respectively, and subjected to SDS-PAGE followed by anti-PVX CP immune-blotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Three plants per genotype were tested in each experiment and the experiment was repeated three and eight times for (A-B) and (C-D), respectively.

2.3.5 An *ago2/ago5* double mutant displays increased susceptibility to PVX compared to single mutants

To validate functional results showing that AGO2 and AGO5 may act synergistically to restrict PVX (Figure 1A to 1D; Supplemental Figure 1A to 1D), we created two independent *ago2 ago5* double mutant lines by crossing the *ago2-1* and *ago5-1* lines and challenging them with PVX. In agreement with our previous report (Jaubert et al., 2011), the *ago5* single mutant behaved the same as WT (Figure 5C), whereas the double mutant plants showed somewhat higher levels of accumulation of PVX in inoculated leaves than the single *ago2* (Figure 5C). Interestingly, this effect appears to have a more pronounced effect in systemic leaves (Figure 5D) suggesting that AGO5 is more important in systemic tissues. Similar results were observed with an independent double mutant made by crossing the *ago2-1* and an additional *ago5* knock out (*ago5-5*; SALK_037270C) line (Supplemental Figure 8A and 8B).

Because luciferase assays indicated a possible antiviral activity of AGO1 and AGO7, we also challenged double and triple mutants, *ago1/2*, *ago2/7* and *ago1/2/7*. None of these mutants was more susceptible to PVX than the single *ago2* mutant at either the local or systemic level and *ago1/2* mutants even appeared somewhat less susceptible to PVX (Figures 5C and 5D, Supplemental Figures 8C and 8D). These results confirm that AGO1 and AGO7 do not play important roles in defense against PVX in *Arabidopsis* and are consistent with *ago1* mutants being more resistant to TRV (Ma et al., 2015).

2.3.6 *AGO5* expression is induced upon PVX infection

Transcriptional analysis monitoring *AGO* gene expression in *Arabidopsis* has indicated that *AGO5* is poorly, if at all, expressed in *Arabidopsis* leaves in unchallenged conditions as well as under biotic and abiotic stresses (Schmid et al., 2005, Borges et al., 2011, Thieme et al., 2012; AtGenExpress Visualization Tool). We therefore monitored *AGO5* expression in uninfected and PVX inoculated plants (WT, *triple dicer*, *ago2* and *ago2*/*ago5*) in both local and systemic tissues. Interestingly, we observed, by RT-PCR, a low level of expression of *AGO5* in the *ago2*

mutant in the absence of PVX. In the other genotypes, we observed expression of *AGO5* mRNA only in PVX infected plants and this, only in the systemic tissues of those genotypes that allow PVX accumulation, with the exception of the *ago2*/*ago5* mutant (Figure 6A). A similar induction was seen by immune-blotting with AGO5-specific antibodies, with AGO5 protein being detected only in the systemic tissues PVX infected plant. Furthermore, AGO5 protein accumulation appeared to correlate with level of PVX accumulation in these genotypes in that the *triple dcl* mutant showed a higher accumulation of AGO5 protein compared to the *ago2* mutant and no AGO5 protein was detected in WT plants. These observations were also confirmed with a transgenic *Arabidopsis* line (P_{AGO5}:GFP-AGO5) expressing GFP-AGO5 from the AGO5 promoter (McCue et al., 2012). Although some AGO5-GFP expression is detected in unchallenged conditions in these plants, expression was significantly enhanced in systemic leaves by PVX infection (Figure 6B). Likewise, upon PVX infection, GFP fluorescence could be detected by microscopy in systemically infected leaves, particularly in the cytoplasm of guard cells (Supplemental Figure 9).

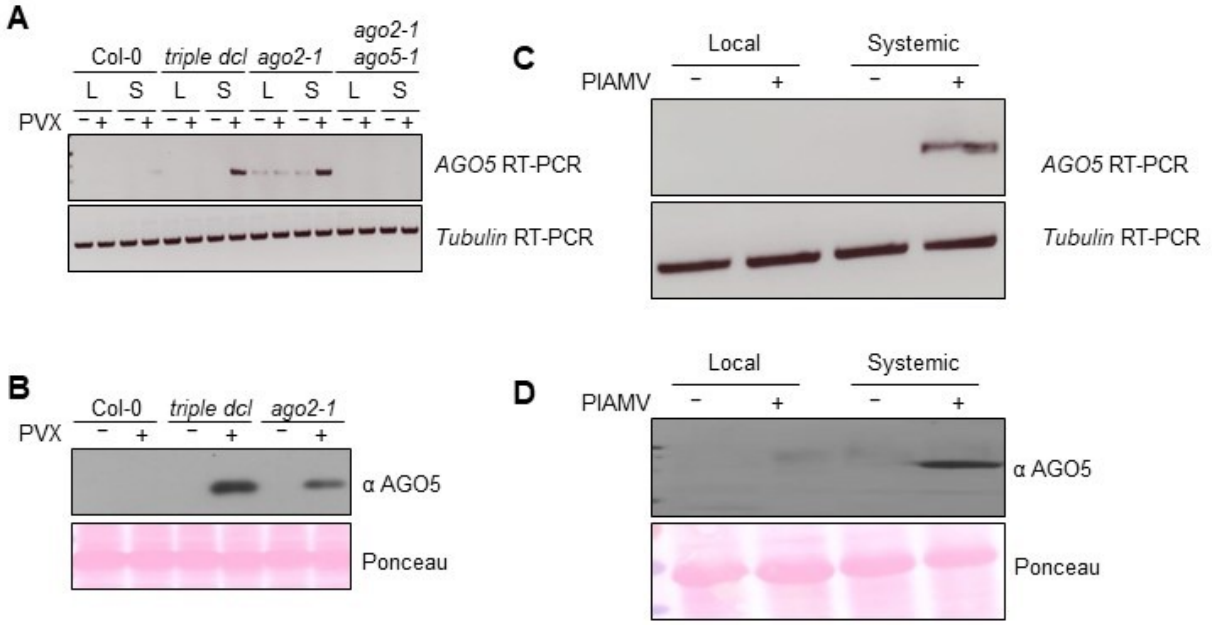

Figure 6. AGO5 expression is induced in *Arabidopsis* systemic leaves upon Potexvirus infection. (A) *Arabidopsis* plants of the indicated genotypes were either mock inoculated or inoculated with PVX (A, B) or PlAMV (C, D). At 7 dpi, total RNA was extracted from

inoculated and systemic leaves, and subjected to RT-PCR with *AGO5*- or *tubulin*-specific primers, as indicated (A, C). Total protein extracts were prepared from inoculated or systemic leaves and subjected to SDS-PAGE followed by anti-AGO5 immunoblotting and Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading (B, D). Data shown are representative of three independent experiments in which at least three separate plants of each genotype were tested.

To determine if other Potexviruses might induce AGO5 accumulation, and to rule out an effect of the mutant backgrounds used above, we challenged *Arabidopsis* Col-0 plants with *Plantago asiatica mosaic virus* expressing GFP (PlAMV-GFP) (Yamaji et al., 2012). Similar to the results observed with PVX, *AGO5* induction was observed in systemic tissues by PlAMV-GFP infection by both RT-PCR and immune-blot analysis (Figure 6 C and 6D). Taken together, these results indicate that AGO5 expression is induced in response to Potexvirus infection and is consistent with the higher susceptibility to PVX observed in the double *ago2*/*ago5* mutant.

2.4 Discussion

The investigation of RNA silencing in anti-viral resistance presents a challenge in that most viruses are already able to overcome this defense response in their host plant. A number of studies have shown that RNA silencing components in antiviral defense can be studied using viruses lacking their cognate VSR (Scholthof et al., 2011; Wang et al., 2011; Garcia-Ruiz et al., 2010; Qu et al., 2008; Diaz-Pendon et al., 2007; Deleris et al., 2006). However, VSRs are often multifunctional proteins involved not only in suppressing RNA silencing (Incarbone and Dunoyer, 2013). As such, their deletion may result in viruses that do not reflect a normal infection and may alter which defenses are effective against it. Indeed, our study showed that there are striking differences between the AGO proteins that can target PVX deletion mutants compared to the wild type virus. Thus, in using a combination of wild type and mutant PVX along with a combination of compatible and incompatible host plants (*N. benthamiana* and *A. thaliana*, respectively), we have shown the importance of viral structures in anti-viral defenses.

Furthermore, we have shown for the first time an essential role for AGO5 in curtailing virus infection in systemic tissues in *Arabidopsis*.

2.4.1 Which Argonautes are antiviral?

Previously, genetic analysis of *AGO* genes in *Arabidopsis* identified AGO1, AGO2, AGO4 and AGO7 as being required for defense against different RNA viruses leading to the hypothesis that only a subset of AGO proteins are specialized in recognition and restriction of RNA viruses. Of the AGO proteins tested (AGOs 1, 2, 5, 7), AGO1, AGO2, and AGO5 have been shown to bind to vsiRNAs by immunoprecipitation analysis in *Arabidopsis* (Takeda et al., 2008; Azevedo et al., 2010). At the same time, studies in *N. benthamiana*, have shown that AGOs 1, 2, 3, 4, 5 and 9 are associated with sRNAs derived from the viroid PSTVd, which to the RNA silencing machinery, may appear much like a virus lacking a VSR (Minoia et al., 2014). *In vitro* experiments also demonstrated the ability of several AGO proteins to target viral transcript in presence of artificial siRNA (Schuck et al., 2013). These observations strongly suggest that the majority, if not all, AGO proteins possess the intrinsic capacity to target viral RNAs. Our results observed upon overexpression of *Arabidopsis* AGO proteins with PVX-GFPΔTGB and PVX-GFPΔP25 (Figure 2 and Supplemental Figure 2) are in agreement with this hypothesis. The latter results are not likely to be due to overexpression as WT PVX is affected only by those AGOs that also show a phenotype in *Arabidopsis* (Figure 1; Figure 5; Supplemental Figure 1; Supplemental Figure 8). Furthermore, this activity was specific to viral RNA as none of the AGO proteins tested affected the accumulation of GFP derived from a 35S:mGFP5 construct (Supplemental Figure 3).

The simplest explanation for the difference between WT and mutant PVX in their sensitivities to different AGOs would be that P25 inhibits all AGO proteins except AGO2 and AGO5. However, in our complementation assays of PVX-GFPΔTGB with P25 expressed *in trans*, we observed that P25 only affected the anti-viral activity of AGO proteins that are targeted by P25 (Supplemental Figure 4), namely AGO1 and AGO7 (Figure 2). In contrast, all other AGO proteins tested, which are not destabilized in the presence of P25, are still efficient in restricting

PVX-GFPΔTGB in the presence of P25 (Figure 2 and Supplemental Figure 4). We observed similar results with PVX-GFPΔP25 (data not shown). This lack of complementation *in trans* may be because a certain stoichiometry between TGB1 (P25) and TGB2/TGB3 is required for the formation of certain structures (Verchot et al., 1998). TGB proteins are required for cell-to-cell movement and for the formation of virus replication factories known as X-bodies, wherein P25 is required to remodel host actin and endomembranes and to recruit TGB2 and TGB3 to the perinuclear X-body (Tilsner et al., 2012). TGB proteins are not required for viral replication, although PVX replication is enhanced by X-bodies, presumably as they serve as scaffolds and protective compartments for virus replication and assembly (Tilsner et al., 2012; Morozov and Solovyev, 2003; Verchot et al., 1998). The lack of X-body formation can be complemented by co-expression of a VSR (Tilsner et al., 2012). Together, these observations suggest that viruses may be protected by VSRs but also by virus-induced subcellular structures that shield them from the RNA silencing machinery. Thus, we propose that to target viruses efficiently, not only must AGO proteins not be inhibited by the virus VSR, but must also be able to access viral RNA. Indeed, both AGO2 and AGO5 are able to bind WT PVX RNA, but AGO9, which can only target "naked" (i.e., unprotected by TGB proteins) viral RNA, does not (Figure 3A and Supplemental Figure 5). The reasons why only certain AGO proteins would be able to access viral RNA remain to be elucidated, but it is likely that it depends both on the types of subcellular structures formed by individual viruses and by the localization properties of different AGO proteins. We speculate that in some cases, for example when a virus possesses a weak VSR, VRC/virus lifestyle may be as important as the VSR activity by itself. Indeed, the P25 protein by itself is a relatively weak VSR in non-virus based VSR assays in *N. benthamiana*, but nonetheless appears to be quite effective in the context of a viral infection in this host (Senshu et al., 2009).

Previous reports have shown a requirement for AGO1 and/or AGO7 in defense against attenuated CMV and TCV (Qu et al., 2008; Morel et al., 2002). However, these two proteins appear to be dispensable for defense against TRV (Ma et al., 2015) and AGO2 is more important than AGO1 in defense against WT TCV (Zhang et al., 2012). Likewise, even though AGO1 and AGO7 are destabilized by the PVX VSR, our genetic and functional analyses suggest that they

are not major determinants of PVX infection. This finding reinforces the importance of using wild type virus in studying plant-virus interactions. At the same time, it may also indicate that different viruses may be affected by different AGO proteins, depending on factors such as replication strategies, VSR mode of action and accessibility of viral RNA to different AGO proteins. For example, a recent report has shown that AGO5 plays only a minor role in defense against TuMV (Garcia-Ruiz et al., 2015). As such, different *ago* mutants may confer divergent phenotypes when infected with various viruses.

2.4.2 Redundant functions for DCL2 and DCL4 in PVX-*Arabidopsis* interaction

Multiple studies have shown that both DCL2 and DCL4 are involved in curtailing systemic infection by TumV, TRV, TCV and CMV (Garcia-Ruiz et al., 2010; Qu et al., 2008; Diaz-Pendon et al., 2007; Dunoyer et al., 2007; Bouche et al., 2006; Deleris et al., 2006). It has been proposed that DCL4 and DCL2 have hierarchical antiviral activities where DCL4 is the primary sensor of viral RNAs. This is based on the observation that in WT plants, VSR-defective TCV RNA appears to be mainly processed by DCL4 but that in its absence, DCL2 is sufficient for antiviral defense (Deleris, et al., 2006). TuMV is likewise preferentially targeted by DCL4, although the double *dcl2*/*dcl4* is more susceptible to TuMV than a *dcl4* single mutant (Garcia-Ruiz et al., 2010). In this study, we found that both *dcl2* and *dcl4* single mutants allow PVX to produce a similar degree of systemic infection, although to a lesser extent than in double *dcl2/dcl4* mutant (Figure 5A and 5B). This result suggests that both DCL2 and DCL4 are required in local and systemic leaves to restrict PVX although DCL2 appear to be less important in local leaves. Our results are similar to a recent report showing that the *dcl2*/*dcl4* mutant is susceptible to PVX, although they did not find that *dcl2* or *dcl4* single mutants were systemically susceptible to PVX (Andika et al., 2015). This may be due to inoculation method or dosage as DCL2 activity may be overcome by increasing inoculum (Deleris et al., 2006) or different growth conditions, which can alter RNA silencing efficiency (Ma et al., 2015; Zhang et al., 2012). Nonetheless, our functional data in *N. benthamiana* are in agreement with our genetic data in *Arabidopsis*. That is, in the presence of VSRs that sequester 21-nt siRNAs, AGO2 still retains a degree of antiviral activity suggesting that AGO2 can utilize both DCL4- and DCL2-

derived siRNAs to target viruses (Figure 4). This is in agreement with a report showing that the survival of TCV-inoculated *Arabidopsis* requires AGO2 programmed by DCL2-produced 22-nt vsiRNAs (Zhang et al., 2012). Thus, given that AGO2 binds 21-nt and 22-nt but not 24-nt sRNAs (Minoia et al., 2014; Mi et al., 2008; Takeda et al., 2008), it would appear that DCL2 and DCL4 have an additive effect in the PVX-*Arabidopsis* interaction.

2.4.3 AGO2 and AGO5 act cooperatively to counteract PVX infection

AGO2 antiviral activity has been demonstrated for several positive-sense ssRNA viruses (Ma et al., 2015; Zhang et al., 2012; Carbonell et al., 2012; Jaubert et al., 2011; Scholthof et al., 2011; Harvey et al., 2011; Garcia-Ruiz et al., 2010) but a major anti-viral role for AGO5 has not previously been shown. Although AGO5 has been shown to bind CMV- and PSTVd-derived siRNAs, such an observation is not informative as most AGO proteins tested bind such sRNAs (Takeda et al., 2008; Minoia et al., 2014). In this report we find that the *ago5* single mutant shows no increased susceptibility to PVX. However, in inoculated leaves, the *ago2*/*ago5* double mutant is more susceptible to PVX than the *ago2* single mutant (Figure 5C and 5D; Supplemental Figure 8A and 8B). This effect is even more pronounced in systemic tissues where the *ago2*/*ago5* double mutant show a much greater susceptibility to PVX than the *ago2* single mutant, similar to that of the triple dicer mutant (Figure 5C and 5D; Supplemental Figure 8A and 8B). We suggest therefore that AGO2 is most important in inoculated leaves, while AGO5 is more important in systemic tissues. Thus, unless AGO2 is absent, PVX cannot move beyond the inoculated leaves, explaining why the lack of phenotype in the *ago5* single mutant. Our finding underlines the utility of studying AGO proteins using functional assays, which can then be followed up with genetic analysis.

A lesser role for AGO5 in inoculated tissues is further supported by the expression profile of *AGO5*, which in uninfected plants, has been shown to be highly specific to reproductive tissues (Schmid et al., 2005, Borges et al., 2011). However, we found that *AGO5* expression is induced in systemic, but not inoculated, tissues after PVX infection. This induction correlated with the degree of susceptibility, being undetectable in Col-0 plants, but detectable in triple dicer and

ago2 mutants, suggesting that a certain threshold of PVX accumulation is necessary to induce *AGO5* expression. This induction is not dependant on the latter mutations as PlAMV, which infects *Arabidopsis* (Yamaji et al., 2012), induced *AGO5* expression in WT Col-0 plant. Interestingly, it has recently been reported that AGO18, a member of a monocot-specific Argonaute protein clade, mediates virus resistance and is induced in virus infected tissues (Wu et al., 2015). Whether systemic induction of *AGO5* expression is induced by the presence of viral RNA itself, secondary vsiRNAs or some other endogenous signal, and whether it shares similarities with *AGO18* induction, remains to be elucidated. However, it is curious to note that *AGO5* mRNA was consistently, albeit faintly, detectable in the leaves of *ago2* mutant plants (Figure 6; Supplemental Figure9), which could indicate that if AGO2 is overcome in inoculated leaves the plant induces AGO5 to counteract a systemic infection.

AGO2 has been shown to bind 21- and 22-nt sRNA, with a preference for 21-nt, while AGO5 binds 21, 22 and 24-nt sRNAs equally (Minoia et al., 2014; Wang et al., 2011; Takeda et al., 2008; Mi et al., 2008). Transient assays with VSRs demonstrated that AGO2 and AGO5 require vsiRNAs for their antiviral function. However, when expressed with P19, which only affects 21-nt sRNAs, AGO2 still possess some antiviral activity, indicating that in the absence of 21-nt sRNAs, it can make use of 22-nt sRNAs, consistent with the additive effects of DCL2 and DCL4 in the PVX-*Arabidopsis* interaction (Figure 4; Figure 5A and 5B). However, given that 21-nt-binding VSRs inhibited the anti-viral activity of AGO5 (Figure 4), this suggests that AGO5 utilizes 21-nt vsiRNAs to target viruses.

2.4.4 AGO2 and AGO5 associate with PVX RNAs

In this study, both wild type and catalytically inactive versions of AGO2 and AGO5 were found to be associated with PVX RNAs using two different methods. Although we do not rule out a possible indirect interaction, given the known modes of action of Argonaute proteins, we suggest that AGO2 and AGO5 bind directly to viral RNAs. Given the requirement for siRNAs for this association (Figure 4C), this binding likely involves an active RISC and may involve additional proteins. The ability of a functional AGO to bind an RNA stably enough for detection

may seem counterintuitive, as it would be predicted to cleave the substrate. However several studies have shown that product release after cleavage by human RISC complexes is the limiting step (Parker et al., 2010, Ameres et al., 2007; Haley et al., 2004; Martinez and Tuschl, 2004). Thus, despite being active, it is still possible to immunoprecipitate AGO-RNA complexes (Wang et al., 2010; Wang et al., 2009; Schwarz et al., 2004).

Although catalytic residues are dispensable for viral RNA binding, they are nonetheless essential for antiviral activity against PVX suggesting that binding viral RNAs is not sufficient to compromise virus accumulation. *In vitro* catalytic assays have shown that AGO2 and AGO5 possess a strong slicer activity against TBSV transcripts (Schuck et al., 2013). This is in contrast with AGO18, which does not appear to directly target viral RNA but rather, functions by sequestering an endgonous miRNA targeting AGO1 (Wu et al., 2015). Our results are however in agreement with a previous study showing that the catalytic activity of AGO2 is required for defense against TuMV (Carbonell et al., 2012). Furthermore, both in this and in a previous study (Carbonell et al., 2012), the presence of catalytically inactive AGO2 increased viral titer suggesting it may compete with WT AGO2 for binding to protein partners and/or RNA (Carbonell et al., 2012) (Figure 3).

2.4.5 A model for AGO2 and AGO5 in anti-viral defenses

We propose a model for defense against potexviruses wherein highly structured or dsRNA is processed by DCL2, 3 and 4 in initially infected cells. DCL4-produced vsiRNAs appear to be most important in these leaves and are likely bound by AGO2, which in turn targets viral RNAs for slicing. If however, the virus is able to overcome AGO2 in inoculated leaves, this appears to initiate the production of an as yet unknown signal that induces the expression of AGO5 in systemic tissues. The reason for the induction of AGO5 in systemic tissues may be because it is involved in utilizing the systemic signal, presumed to include vsiRNAs, to target viruses (Parent et al., 2014; Mourrain et al., 2000; Voinnet et al., 2000). DCL2 and DCL4 however, appear to play equally important roles in systemic infections and it remains to be seen is this is due to their roles in the systemic tissues or in the production of the systemic signal. If this signal is able to

move more quickly than the virus into systemic tissues, then we speculate that it may be incorporated into AGO5-containing RISC complexes in uninfected tissues. Thus, this second line of defense could target incoming viral RNAs before they are able to establish an infection in systemic tissues. Differences in the requirement for AGO5 in defense against specific viruses, such as TuMV (Garcia-Ruiz et al., 2015), could be due to differences in the production of, or susceptibility to, the systemic silencing signal. The importance of systemic signals in PVX infection is underlined by the fact that the PVX VSR, P25 functions by inhibiting the systemic movement of the silencing signal in *N. benthamiana* (Voinnet et al., 2000). The PVX P25 protein has not been demonstrated to inhibit this aspect of RNA silencing in *Arabidopsis*, whereas the P25 protein of PlAMV is thought to do so (Okano et al., 2014). Given that AGO2 seems to be involved in most plant-virus systems tested, this model is likely to extend to other viruses. However, the involvement of AGO5 may vary between viruses depending on the mode of action of their VSRs and their movement strategies.

2.5 Methods

2.5.1 Plant material and growth conditions

Nicotiana benthamiana and *Arabidopsis thaliana* plants were grown on soil (BM6, Fafard and Agromix, PLACE, respectively) in growth chambers with 16-h-light/8-h-dark photoperiod at 23°C and 21°C respectively. Except for the pAGO5:AGO5-GFP transgenic line, which is in Ler background (McCue et al., 2012), all *Arabidopsis* mutant lines were of the Col-0 ecotype and have been previously described, including the *ago1-27* (Morel et *al.*, 2002), *ago2-1* (Takeda et *al.*, 2008), *ago5*-1 (Katiyar-Agarwal et *al.*, 2007; Mi et al., 2008), ago1/ago2, ago1/ago2/ago7, ago2/ago7 (Wang et *al.*, 2011), *triple dicer* (Deleris et al., 2006), *dcl1-9* (Jacobsen et al., 1999), *dcl2*-1 and *dcl3-1* (Xie et al., 2004) and *dcl4-2* and *dcl2/dcl4* (Xie et al., 2005).

The *ago2/ago5* mutant lines were generated by standard genetic crosses between homozygous *ago2-1* mutants (Salk_003380) and either the *ago5-1* mutant (Salk_ 063806) or the *ago5-5*

mutant (Salk_037270C). Homozygous double mutant genotypes were confirmed by allele-specific PCR at the second generation.

2.5.2 Plasmid construction

Construction of all pBIC-HA-AtAGO has been described previously (Takeda et *al.*, 2008). For the generation of pBIN61-FLAG-AGO2, pBIC-HA-AGO constructs were used as templates for PCR amplification using KOD high fidelity DNA polymerase (Novagen). Primer sequences are listed in Supplemental Table 1 online. PCR fragments were then A-tailed with Taq DNA polymerase (New England Biolab) and cloned into pGEM-T easy vector (Promega) for sequencing. Inserts were then digested and cloned into the *Xba* I and *Bam* HI sites of pBIN61-FLAG. 35S:HA-AGO2 and 35S:HA-AGO5 slicer-defective variant were generated by PCR mutagenesis using pBIC-HA-AGO2 and pBIC-HA-AGO5 as templates. Primers used are listed in Supplemental Table 1.

PVX, PVX-GFP and PVX-GFPΔTGB binary constructs (Peart et al., 2002 and Bhattacharjee et al., 2009), PlAMV-GFP (Yamaji et al., 2012); 35S:P14, 35S:P21 (Mérai et al., 2006), 35S:P15 (Dunoyer et al., 2002) and 35:P19 (Voinnet et al., 1999) have been previously described.
pBIN61-P25:HA was generated by RT-PCR from PVX-infected plants using primers (Supplemental Table 1) to introduce *Xba* I and *Bam* HI sites at the 5' and 3' ends of the P25 ORF, respectively. The resulting PCR fragment was the cloned into the same sites of pBIN61:FLAG (Moffett et al., 2002) and verified by sequencing.

Firefly luciferase was amplified from Luciferase T7 Control DNA (Promega) with specific primers (Supplementary Table 1) and cloned into the *Asc* I and *Sal* I sites of pGR106 (Jones et al., 1999). To generate the 35S:R-LUC-expressing construct, the pGreenII61 MCS (including the 35S expression cassette) was first subcloned into the *Asc* I and *Stu* I sites of pEAQ-SelectK (Sainsbury et al., 2009) to produce the pEAQ-SE expression vector (Ali et al., 2015). Subsequently, Renilla luciferase was excised from pRL-SV40 (Promega) with NheI and *Xba* I and cloned into the *Xba* I site of pEAQ-SE. Insert orientation was verified by sequencing.

2.5.3 Virus inoculation

Infections of three-week-old *Arabidopsis* plants were carried out by rub inoculation as previously described (Jaubert et al., 2011). Briefly, saps were produced with PVX-infected or PlAMV-GFP-infected *N. benthamiana* plants by grinding infected tissue in in 0.1M phosphate buffer, pH 7.0 (2 ml/g of infected tissues). Mock infections were carried out with sap produced with uninfected *N. benthamiana* plants (2 ml/g of healthy tissues).

2.5.4 Transient expression assays

Agrobacterium-mediated transient expression assays in *N. benthamiana* were performed as previously described (Moffett, 2011). Briefly, binary expression constructs were transformed into the C58C1 *Agrobacterium tumefaciens* strain carrying pCH32 virulence plasmid. For virus agroinoculation, GV3101 *Agrobacterium tumefaciens* strain carrying the pSoup helper plasmid was transformed with pGr106/pGr107/pGr208 derivatives constructs (PVX-GFP WT, PVX-GFPΔTBG, PVX-GFPΔP25, PVX-LUC, PVX-LUCΔTBG, PVX-S1). *Agrobacterium tumefaciens* cultures were centrifuged at 4,000 RPM for 10 min and resuspended in 10 mM $MgCl_2$ to a final OD600 = 0.2 and 0.1 for protein expression and virus vectors respectively.

2.5.5 Protein extraction and analysis

For AGO expression analysis, 1 g of fresh tissues was ground into 2 mL of RISC buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 0,5% NP-40) supplemented with protease inhibitor cocktail. Total protein extract was centrifuged at 16 000 xg for 10 minutes at 4°C. A fraction of total protein extract was kept for detection of GFP. Immunoprecipitation was carried out with 1.4 ml of supernatant and 25 µl of HA-agarose beads (Sigma) or FLAG-agarose beads (Sigma) for 2 h at 4°C on a rotatory shaker. Beads were washed 4 times with RISC buffer. After centrifugation, beads were resuspended in 50 µl of 1.5X of Laemmli loading buffer. Proteins were separated by SDS-PAGE on 7.5% or 10.5% acrylamide

gels for AGO or P25, GFP and CP detection respectively, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by electroblotting. HA-AGO proteins were probed with anti-HA-horseradish peroxidase conjugated (HRP) antibodies (Sigma, 1:3,000 dilution). FLAG tagged proteins were probed with anti-FLAG-HRP antibodies (Sigma, 1:5,000 dilution). Detection of GFP was carried out by probing membranes with anti-GFP-HRP antibodies (Santa Cruz, 1:3,000 dilution). Anti-PVX-CP rabbit polyclonal antibodies (Agdia, 1:3,000 dilution) were used to detect PVX in *Arabidopsis* followed by donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend, 1:10,000 dilution). Detection of *Arabidopsis* AGO5 was carried out by probing membranes with anti-AGO5 antibody (Agrisera, 1:3000 dilution) and subsequently with donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend, 1:10,000 dilution). Equal loading of proteins was confirmed with polyclonal antibody phosphoenol pyruvate carboxylase (anti-PEPC, Rockland; 1:10,000 dilution).

2.5.6 RNA immunoprecipitation

Analyses of AGO-RNA interactions were performed as described previously (Carbonell et al., 2012), with some modifications. Briefly, *Agrobacterium* infiltrated *N. benthamiana* leaves were ground with mortar and pestle in cold extraction buffer 2ml/g (50 mM Tris-HCl, pH7,4, 2.5 mM $MgCl_2$, 100 mM KCl, 0.1% Nonidet P-40) supplemented with protease inhibitor cocktail and ribolock 40 units/ml (ThermoScientific), followed by centrifugation at 16,000 xg for 10 minutes at 4°C. Total RNA (aliquots of 250 µl) was used for input controls. A pre-clearing step was performed on 5 ml of supernatant by adding 35 µl of non-specific IgG-agarose beads (Rockland Immunochemicals) for 30 min at 4°C on a rotatory shaker. After spinning down beads, supernatant was incubated with 75 µl of anti-HA agarose beads (Sigma) for 2 hours at 4°C. Beads were washed eight times with 5 ml of extraction buffer. AGO-associated RNAs and proteins were isolated from beads with Trizol, as per the manufacturer's instructions (Ambion).

2.5.7 Dual-Luciferase reporter assay

Four days after agroinoculation, three leaf discs were ground in 100 µL of passive lysis buffer (Promega). After centrifugation, 20 µl of protein extract of each sample was distributed in triplicates in 96-well plates and dual-luciferase reporter assay was performed in accordance with the manufacturer's protocol (Promega).

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2.7 Author contributions

C.B. and P.M. designed the research, analyzed the data and wrote the manuscript. C.B. performed the experiments. Both authors read, helped to edit, and approved the final version of the manuscript.

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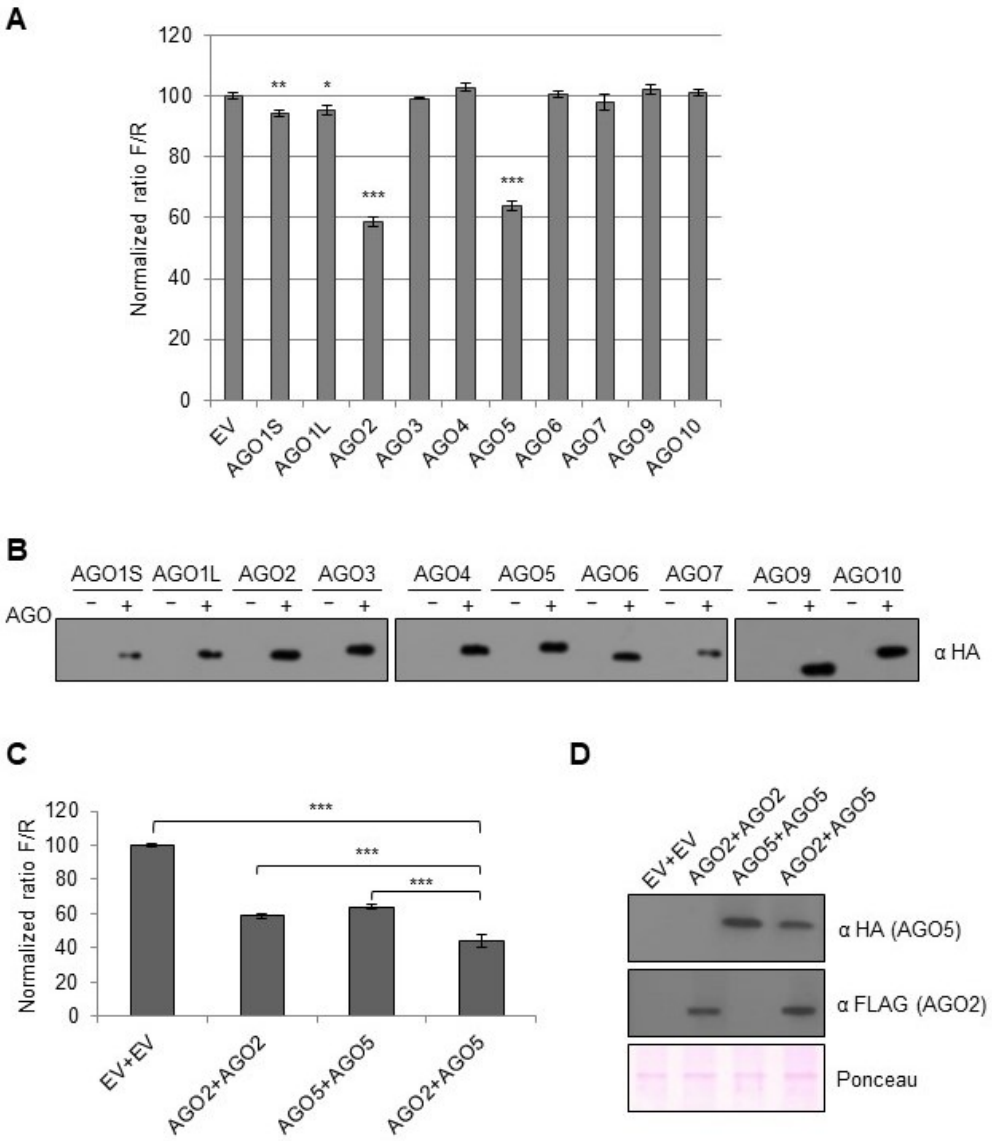
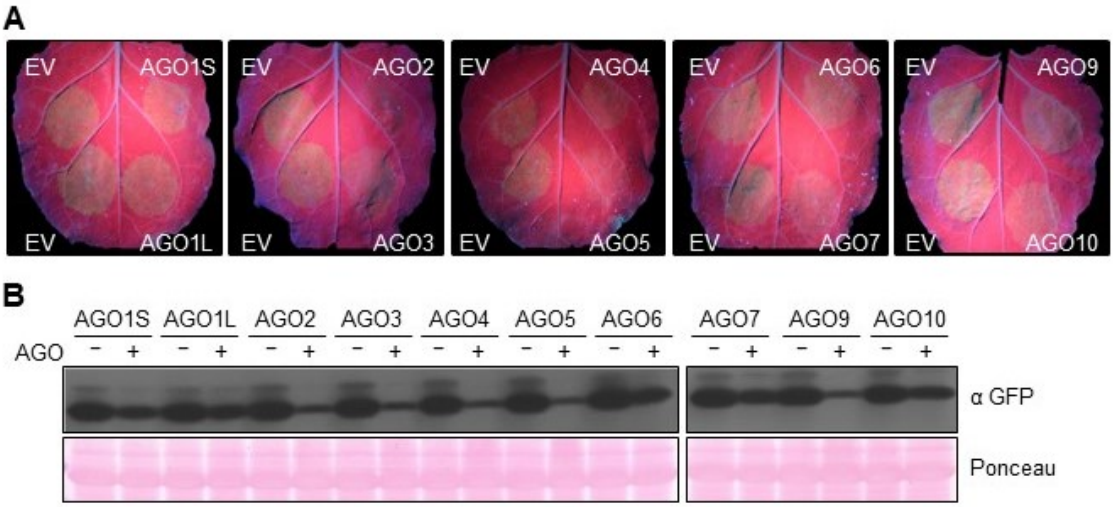
2.8 Supplemental Data

2.8.1 Supplemental Figures

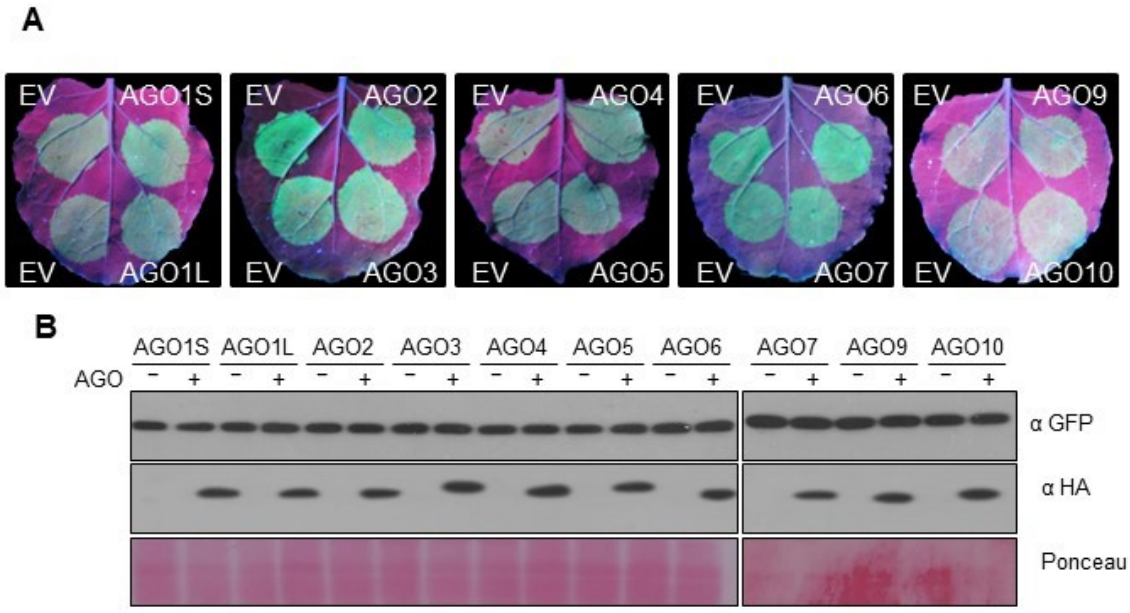


Supplemental Figure 1. AGO2 and AGO5 act synergistically to counteract PVX accumulation in *N. benthamiana*. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-LUC and 35S:R-LUC along with ten different HA-tagged *Arabidopsis* AGO proteins or empty vector (EV), as indicated. Luciferase activities were measured from protein extracts prepared from infiltrated tissues 4 dpi. Bar plots represent F-LUC activity normalized to R-LUC activity.

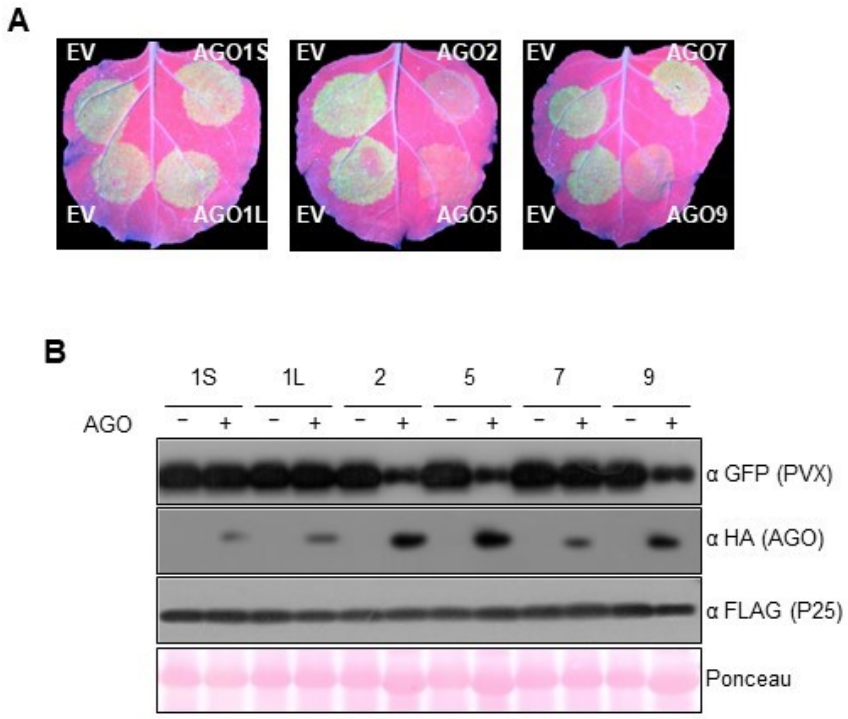
Values represent means ± SEM from three independent experiments (*n*=9). Data sets marked with one, two or three asterisks are significantly different from empty vector infiltrated leaves as assessed by Student's *t* test at P-value < 0.05, 0.005 or 0.0001, respectively. (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) at 4 dpi. HA-tagged AGO proteins were immunoprecipitated and subjected to anti-HA immunoblotting. (C) *N. benthamiana* leaves were agroinfiltrated with PVX-LUC and 35S:R-LUC along with EV, FLAG-AGO2 or HA-AGO5 alone or in combination, as indicated. Luciferase activities were measured from protein extracts prepared from infiltrated tissues 4 dpi. Bar plots represent F-LUC activity normalized to R-LUC activity. Values represent means ± SEM from three independent experiments (*n*=9). Data sets marked with three asterisks are significantly different as assessed by Student's *t* test at P-value < 0.0001. (D) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (C) 4 dpi, and subjected to anti-HA and anti-FLAG immunoprecipitation (IP) followed by anti-FLAG and anti-HA and immune-blotting, respectively.



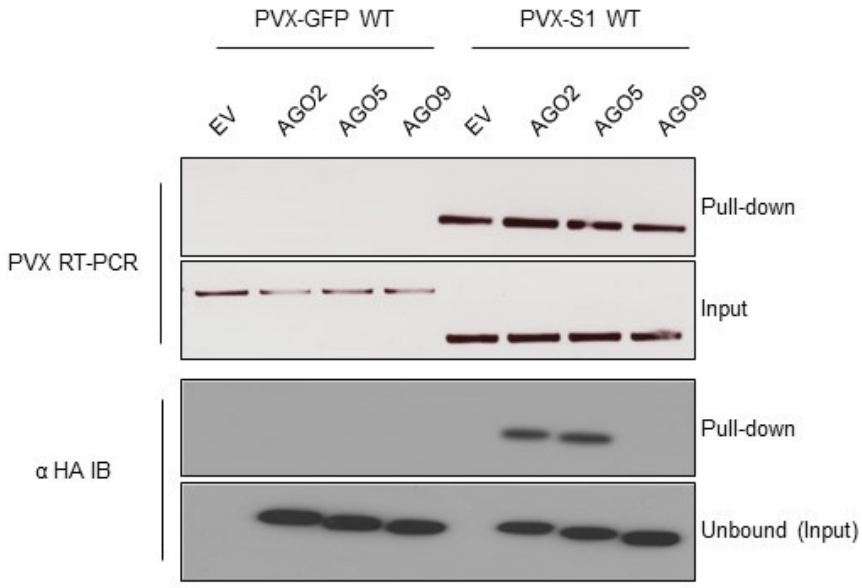
Supplemental Figure 2. All *Arabidopsis* Argonaute proteins can target viral RNA. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFPΔP25 along with either empty vector (EV) or HA-tagged *Arabidopsis* AGO proteins, as indicated. (A) Leaves were photographed under UV illumination 4 days post infiltration (dpi). (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). (+) indicates that presence of the indicated AGO protein and (-) indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.



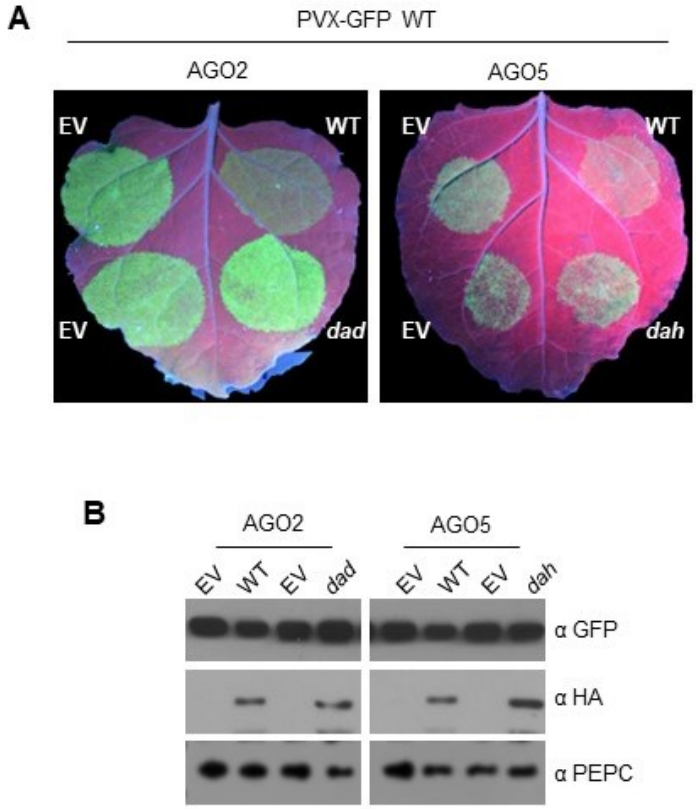
Supplemental Figure 3. Overexpression of *Arabidopsis* AGO does not compromise GFP accumulation from a 35S:GFP construct. (A) *N. benthamiana* leaves were agroinfiltrated with 35S:mGFP5 along with ten different HA-tagged AGO proteins from *Arabidopsis* or empty vector (EV), as indicated. Leaves were photographed under UV illumination 3 dpi. (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 3 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). (+) indicates that presence of the indicated AGO protein and (-) indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Representative pictures are shown of experiments performed six times with at least three plants per treatment.



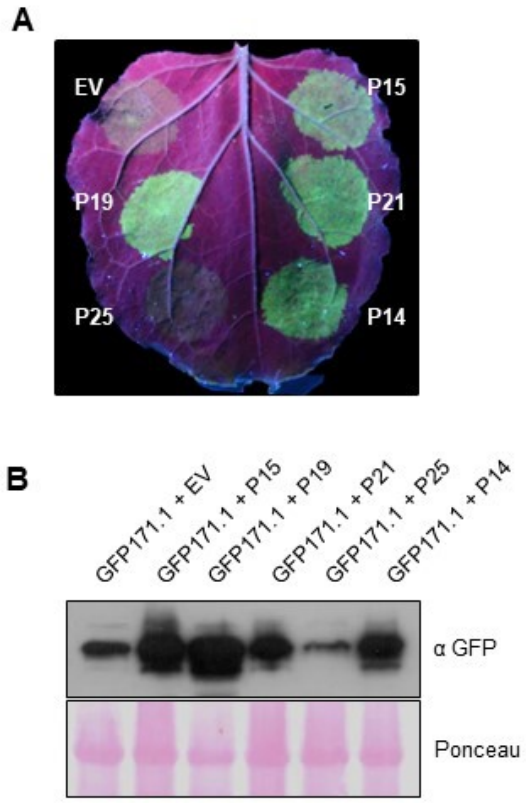
Supplemental Figure 4. Expression in *trans* of P25 compromise antiviral activity of AGO1 and AGO7. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFPΔTGB and FLAG-tagged P25 along with either empty vector (EV) or HA-tagged *Arabidopsis* AGO proteins, as indicated. Leaves were photographed under UV illumination 4 days post infiltration (dpi). (A) Leaves were photographed under UV illumination 4 days post infiltration (dpi). (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel) or anti-FLAG immunoblotting (second panel). (+) indicates that presence of the indicated AGO protein and (-) indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (third panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.



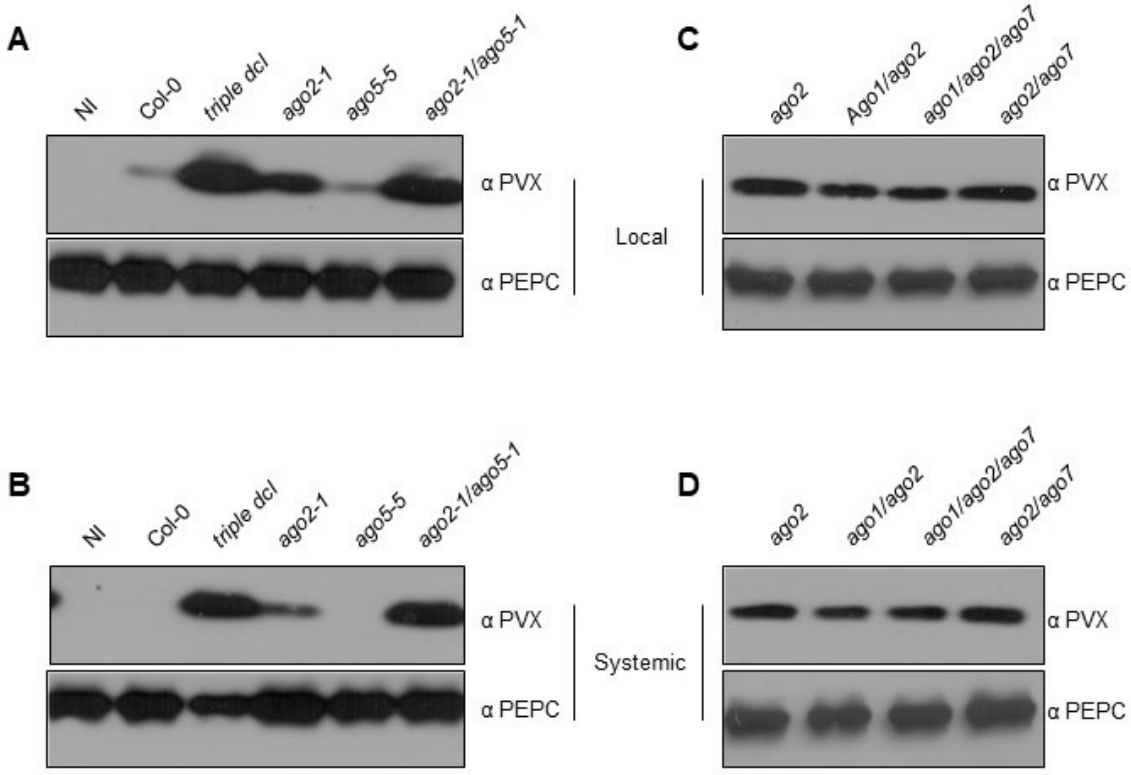
Supplemental Figure 5. AGO2 and AGO5 bind PVX RNAs. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFP or PVX-S1 (S1: encoding a streptavidin-binding aptamer) along with EV, AGO2, AGO5 or AGO9, as indicated. PVX-S1 RNAs were isolated from total extracts at 3 dpi using streptavidin-agarose beads. (A) PVX RNAs were detected in input and pull-down fractions by RT-PCR with PVX specific primers. (B) Proteins bound to isolated PVX RNAs were subjected to SDS-PAGE followed by anti-HA immunoblotting. Data shown are representative of experiments performed three times with similar results.



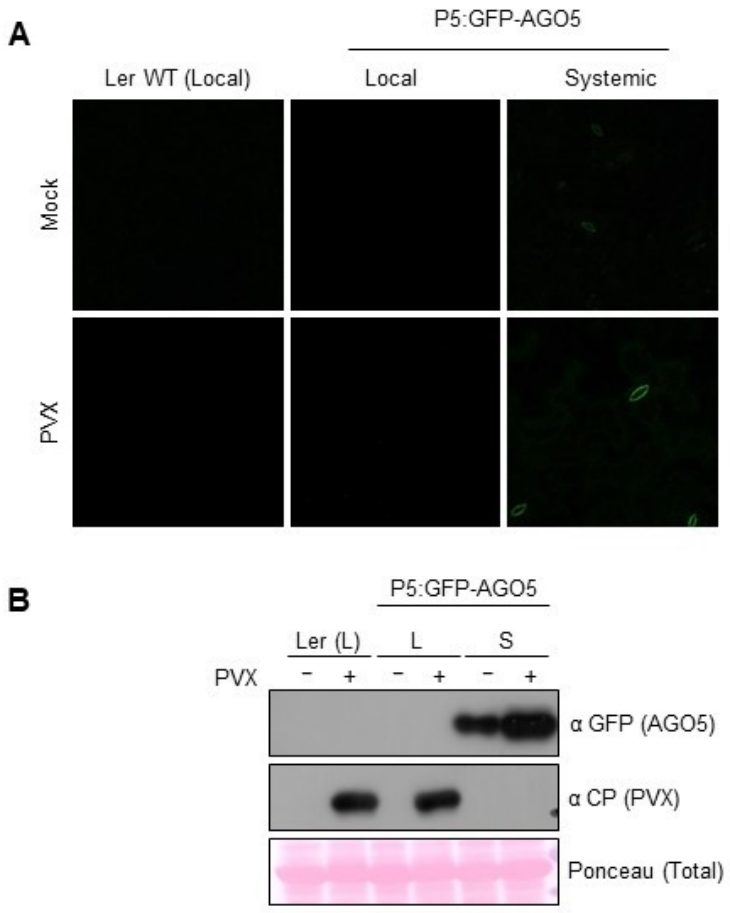
Supplemental Figure 6. Catalytic residues of AGO2 and AGO5 are required to target PVX-GFP. (A) *N. benthamiana* leaves were agroinfiltrated with PVX-GFP along with either EV or WT or catalytically inactive AGO2 or AGO5, as indicated. Leaves were photographed under UV illumination 4 dpi. (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) at 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Anti-PEPC immunoblotting (bottom panel) of the same extracts is shown to demonstrate equal loading.



Supplemental Figure 7. Validation of VSR expression - Viral suppressors of RNA silencing compromise silencing in a transient assay. (A) *N. benthamiana* leaves were agroinfiltrated with a 35S:GFP construct containing a binding site in its 3' UTR for the endogenous miRNA 171.1 along with EV or the VSRs, P14, P15, P19, P25 and P21. Leaves were photographed under UV illumination 4 dpi. (B) Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.



Supplemental Figure 8. PVX infection in an additional *ago5* mutant line and various double mutant lines. Col-0 WT *Arabidopsis* as well single, double or triple *ago* mutant lines, as indicated, were inoculated with PVX. At 5 dpi (A, C) and 21 dpi (B, D) total protein extracts were prepared from inoculated and systemic leaves, respectively and subjected to SDS-PAGE followed by PVX anti-CP immune-blotting (top panel). Anti-PEPC immunoblotting (bottom panel) of the same extracts is shown to demonstrate equal loading. At least three plants per genotype were tested in each experiment and the experiment was repeated three times.



Supplemental Figure 9. Expression of AGO5 is induced by PVX infection in P_{AGO5}:GFP-AGO5 transgenic plants. (A) Ler WT *Arabidopsis* as well as Ler transgenic PAGO5:eGFP-AGO5 plants were either mock inoculated or inoculated with PVX. (A) At 7 dpi, eGFP-AGO5 fluorescence was observed by confocal microscopy (B). Total protein extracts were prepared from local (L) inoculated or systemic (S) leaves and subjected to SDS-PAGE followed by anti-GFP immunoblotting (top panel) or anti-CP immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Five plants per genotype were tested in each experiment and the experiment was repeated five times.

2.8.2 Supplemental Table

Supplemental Table 1. Oligonucleotides used in this study

Name	Sequence (5'→3')	Use
AtAGO2-XbaI-Fwd	TCTAGAATGGAGAGAGGTGGTTATCG	Cloning (pBIN61-FLAG)
AtAGO2-SalI-Rev	TACGTAGACGAAGAACATAACATTCTC	Cloning (pBIN61-FLAG)
LUC For AscI	GGCGCGCCATGGAAGACGCCAAAAACATAAAG	Cloning (PVX-F-LUC)
LUC Rev SalI	GTCGACTTACAATTTGGACTTTCCGCCC	Cloning (PVX-F-LUC)
XbaI25KFor	CCCTCAGAATGGATATTCTCATCATTAG	Cloning
BamHI25KRev	CCGGATCCTGTCCCTGCGCGGACATATG	Cloning
Delta25For	TGACTGAATGAGATTACTTAATTAACGCGTGGCC	Cloning (PVX-GFPΔP25)
Delta25Rev	ACGCGTTAATTAAGTAATCTCATTCAGTCAGGCC	Cloning (PVX-GFPΔP25)
CP-Fwd	AGTAGCCAGCAATGCTGTCG	RT-PCR
CP-Rev	TTGTGCTTGCCAGTTAGCAGG	RT-PCR
Tub-Fwd	GTTCAATGCTGTTGGTGGTG	RT-PCR
Tub-Rev	GTCACACTTTGCCATCATGC	RT-PCR
AGO2-dad-F	TGTGATATTCCGTGCTGGTGTCAGCGATGC	Cloning (mutagenesis)
AGO2-dad-R	GCATCGCTGACACCAGCACGGAATATCACA	Cloning (mutagenesis)
AtAGO2-HA-FwdXbaI	TCTAGA GCC ACC ATG TAC CCA TAC GAT GTT CCT GAC TAT GCG ATGGAGAGAGGTGGTTATCG	Cloning
AtAGO2-RevBamHI	GGATCCTCAGACGAAGAACATAACAT	Cloning
AtAGO5D721A-Fwd	CATCATGGGTGCTGCTGTGACTCACC	Cloning (mutagenesis)
AtAGO5D721A-Rev	GGTGAGTCACAGCAGCACCCATGATG	Cloning (mutagenesis)
HA-AtAGO5-fwd	CCTAGGGCCACCATGTACCCATACGATGTTCCTGACTATGCGTCTAGAATGTCAAATCGTGGTGGTGG	Cloning
AGO5/SalI Reverse	GTCGACTTAGCAATAAAACATAACCT	Cloning

AtAGO5-RT3-Fwd	ACTTCGACGGAGACGAAACC	RT-PCR
At-AGO5-RT3-Rev	AAACGGTCTGTCCTTCCCAG	RT-PCR
pgR106/107-Fwd	CTAGATGCAGAAACCATAAGG	RT-PCR
pgR106/107-Rev	GAGGTAGTTGACCCTATGG	RT-PCR
Streptavidin Aptamer - Fwd	GGGAGTCGACCGACCAGAATCA TGCAAGTGCGTAAGATAGTCGCG GGCCGGGGGCGTAT TATGTGCGTCTACATCTAGACTC AT	Cloning
Streptavidin Aptamer - Rev	ATGAGTCTAGATGTAGACGCACA TAATACGCCCCCGGCCCGCGAC TATCTTACGCACTTGCATGATTCT GGTCGGTCGACTCCC	Cloning

2.8.3 Supplemental Methods

2.8.3.1 Isolation of PVX RNAs

PVX RNAs isolation was performed as described previously (Srisawat and Engelke, 2001), with some modifications. Total extracts were prepared by grinding 3 g of tissue in 6 ml of lysis buffer (50 mM HEPES, pH7.4, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X100, 10% glycerol) supplemented with 1X protease inhibitor cocktail. The homogenate was filtered through a double layer of Miracloth. The flow-though was centrifuged at 16 000 xg for 10 min at 4°C. Total RNA (aliquots of 250 µl) was used for input controls and remaining soluble cell extract was pre-incubated with 50 µl of avidin (from egg white)-agarose beads (Sigma) for 20 min at 4°C on a rotatory shaker. After spinning down beads, supernatant was incubated with 100 µl of streptavidin-agarose beads for two hours at 4°C. The beads were washed eight times with 5 mL of lysis buffer. RNAs and proteins were isolated from beads with Trizol, as per the manufacturer's instructions (Ambion).

2.8.3.2 RT-PCR

Total, immunoprecipitated and pull-down RNA was extracted from samples using the Trizol, according to the manufacturer's instructions (Ambion) and treated with DNAase free RNAase

(QIAGEN). First-strand cDNA was synthesized from 1 µg for total RNA or 12.5 µl for immunoprecipitated and pull-down RNA using M-MLV reverse transcriptase (Promega). Targets (PVX, tubulin or AGO5) were amplified by using primers as listed in Supplementary Table 1.

2.8.3.3 Plasmid construction

Plasmids 35S:mGFP5 and 35S:GFP171.1 have been described elsewhere (Voinnet et al., 2000; Parizotto et al., 2004; Brodersen et al., 2008). PVX-GFPΔP25 was created by insertion of a double stranded oligonucleotide containing stop codons in all three open reading frames into the *Apa* I site of PVX-GFP (Peart et al., 2002). Similarly, PVX-S1, containing streptavidin aptamer, was created by insertion of streptavidin aptamer double stranded oligonucleotide into the *Sma* I restriction site of pGr107 (Lu et al., 2003). Oligonucleotides used are listed in supplemental Table 1.

2.8.3.4 Confocal microscopy

Seven days after rub inoculation, local inoculated or systemic leaf tissue was mounted between slide and coverslip and GFP-AGO5 fluorescence was monitored with a 40X objective, using a 488nm argon laser. Emitted fluorescence was collected using a 510-530 band-pass filter and a 575-630 band-pass on an Olympus FV300 confocal microscope. Each image represents a Z stack of 0.5µm.

2.8.4 Supplemental References

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Peart JR, Cook G, Feys BJ, Parker JE, Baulcombe DC (2002) An EDS1 orthologue is required for N-mediated resistance against tobacco mosaic virus. The Plant journal : for cell and molecular biology 29 (5):569-579.

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CHAPITRE 3

AGO4 EST IMPLIQUÉE DANS LA DÉFENSE ANTIVIRALE LORS D'UNE INTÉRACTION ARABIDOPSIS-POTEXVIRUS

Nous avions préalablement démontré l'implication des protéines AGO2 et AGO5 dans l'immunité antivirale chez *Arabidopsis* contre un virus du genre des Potexvirus, soit PVX. Dans le présent manuscrit, nous voulions déterminer si l'activité antivirale des deux protéines susmentionnées était aussi impliquée dans la répression de PlAMV, autre virus faisant aussi partie du genre Potexvirus. De façon intéressante, nous démontrons que bien qu'AGO2 et AGO5 possèdent une activité antivirale contre ce virus, la répression de ce dernier chez *Arabidopsis* dépend majoritairement de la protéine AGO4. Ces travaux apportent des pistes d'étude sur comment une protéine majoritairement localisée et active au noyau peut arriver à réprimer un virus à ARN, dont le génome et les protéines sont majoritairement localisés au cytoplasme.

Pour cet article, CB, MEO et PM ont conçu les expériences. En tant que premier auteur, CB a réalisé une grande partie des expériences (Figure 1, Figure 2a, b, c,e, Figure 3a, Figure 4, Figure 5a, Figure 6 et les Figures supplémentaires). CB et MEO ont réalisé conjointement les essais d'infection pour les tableaux 1 et S1. MEO a réalisé les expériences pour la Figure 3b. AA a réalisé les expériences pour la Figure 5b. XM a réalisé l'immunobuvardage de type Northern pour la Figure 5d. La rédaction a été faite par CB et PM. AA a révisé et commenté le manuscrit avant la soumission. Cet article a été publié dans la revue scientifique *MPMI* : Brosseau et al., (2016) Antiviral Defense Involves AGO4 in an Arabidopsis-Potexvirus interaction. MPMI *29*, 878-888.

3. ANTIVIRAL DEFENSE INVOLVES AGO4 IN AN ARABIDOPSIS-POTEXVIRUS INTERACTION

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3.1 Summary

In plants, RNA silencing regulates gene expression through the action of Dicer-like (DCL) and Argonaute (AGO) proteins *via* micro RNAs and RNA-dependent DNA methylation (RdDM). In addition, RNA silencing functions as an anti-viral defense mechanism by targeting virus-derived double stranded RNA. Plants encode multiple AGO proteins with specialized functions, including AGO4-like proteins, which effect RdDM and AGO2, AGO5 and AGO1, which have antiviral activities. We show here that *AGO4* is also required for defense against *Plantago asiatica mosaic potexvirus* (PlAMV), most likely independent of RdDM components such as DCL3, Pol IV and Pol V. Transient assays showed that AGO4 has direct anti-viral activity on PlAMV and, unlike RdDM, this activity does not require nuclear localization of AGO4. Furthermore, although PlAMV infection causes a decrease in *AGO4* expression, PlAMV causes a change in AGO4 localization from a largely nuclear to a largely cytoplasm distribution. These results indicate an important role for AGO4 in targeting plant RNA viruses as well as demonstrating novel mechanisms of regulation of, and by, AGO4, independent of its canonical role in regulating gene expression by RdDM.

3.2 Introduction

Plants have multiple mechanisms to defend themselves against viruses, including RNA silencing. RNA silencing encompasses a number of related processes that regulate the expression of endogenous genes as well as foreign nucleic acids, such as viruses, through the recognition and processing of double-stranded RNA (Pumplin and Voinnet, 2013). The major protein effectors of RNA silencing include Dicer-like (DCL) and Argonaute (AGO) endoribonucleases as well as RNA-dependent RNA polymerases (RDR) (Seo et *al*., 2013). In *Arabidopsis*, four different DCL proteins recognize dsRNA and cleave it into short RNA (sRNA) fragments of 21 to 24-nt. DCL1 recognizes mainly dsRNA encoded by endogenous transcripts that form hairpins. DCL1 cleaves these precursors into micro RNAs (miRNAs), which are involved in regulating cellular transcripts. DCL2, DCL3 and DCL4 generally

recognize longer dsRNA molecules originating from endogenous or exogenous sources and process them into small interfering RNAs (siRNAS) (Axtell, 2013; Bologna and Voinnet, 2014). After cleavage of dsRNA, sRNAs are incorporated into RNA-induced silencing complexes (RISC). The core RISC activity is carried out by members of the AGO protein family, which bind the sRNAs and use them as guides to target single stranded RNA with sufficient homology to allow base pairing. In most cases, in plants, this binding results in cleavage of the target ssRNA by the RNase H-like activity of the AGO protein (Borges and Martienssen, 2015), although in some cases, this may result in translational repression, the more common mode of action of AGO proteins in animals (Brodersen et *al.*, 2008). The *Arabidopsis* genome encodes ten AGO family members, a number of which show highly specific expression patterns and subcellular localizations, suggesting specialization in function (Havecker et *al*., 2010; Mallory and Vaucheret, 2010; Borges and Martienssen, 2015). In particular, members of the AGO4 clade (AGO4, AGO6, AGO9) differ from other family members in that they predominantly localize to the nucleus, where they bind to 24-nt heterochromatic siRNAs (hcRNAs) corresponding to repetitive elements of the genome (Zilberman et *al*., 2003; Havecker et *al*., 2010). These hcRNAs are derived from transcription by RNA Pol V and Pol IV, conversion to dsRNA by RDR2 and dicing by DCL3. AGO4-like proteins then recruit proteins involved in DNA methylation and induce RNA-directed DNA methylation (RdDM), resulting in chromatin modification and alteration of gene expression at specific targeted loci (Matzke et *al*., 2015). This pathway has been shown to be implicated in plant defense responses by reprogramming host gene expression (Coego et *al*., 2005; Agorio and Vera, 2007; López et *al*. 2011; Yu et *al*., 2013).

RNA viruses produce dsRNA as a replication intermediate, thus making them targets of RNA silencing (Harris et *al*., 2015), mediated largely by DCL2 and DCL4, which generate virus-derived siRNAs (vsiRNAs) that can be incorporated into RISC complexes (Deleris et *al*., 2006; Bouche et *al*., 2006; Diaz-Pendon et *al*., 2007; Dunoyer et *al*., 2007; Qu et *al*., 2008; Garcia-Ruiz et *al*., 2010; Andika et *al*., 2015; Brosseau and Moffett, 2015). The resulting "aberrant" viral RNA cleavage products are thought to be substrates for plant RDR proteins, which subsequently generate more dsRNA, thus acting as an amplification cycle. The major

contributors to the production of secondary vsiRNAs are RDR1, RDR2 and RDR6 (Zhang et *al*., 2015).

Determining which AGO proteins effect defenses against RNA viruses has been the subject of a number of studies. In an *in vitro* assay AGO1, 2, 3 and 5 all show activity against a Tombusvirus and AGO1, 2, 3, 4, 5, and 9 can bind to sRNAs derived from viroids or viruses (Takeda et *al*., 2008, Wang et *al*., 2011; Schuck et *al*., 2013; Minoia et *al*., 2014). These results suggest that multiple AGO proteins have the intrinsic ability to target viruses. Several AGO mutants show increased susceptibility to viruses (Carbonell and Carrington, 2015), including *ago1*, which are more susceptible to *Cucumber mosaic virus* (CMV), *Turnip crinkle virus* (TCV) and *Brome mosaic virus* (BMV), (Morel et *al*., 2002; Qu et *al*., 2008; Azevedo et *al*., 2010; Dzianott et *al*., 2012) and an *ago7* mutant shows higher accumulation of certain derivatives of TCV (Qu et *al*., 2008). AGO2 appears to be a major player in RNA silencing against viruses and has been implicated in defense against CMV, TCV, TRV, *Potato virus X* (PVX), *Turnip mosaic virus* (TuMV), and *Tomato bushy stunt virus* (TBSV) (Harvey et *al*., 2011; Wang et *al*., 2011b; Zhang et *al*., 2012; Ma et *al*., 2015; Jaubert et *al*., 2011; Carbonell et *al*., 2012; Scholthof et *al*., 2011; Odokonyero et *al*., 2015). In addition, AGO5 appears to play a secondary antiviral role in the absence of AGO2 (Garcia-Ruiz et *al*., 2015; Brosseau and Moffett, 2015) and *ago4* mutants are more susceptible to TRV (Ma et *al*., 2015; Fernandez-Calvino et al., 2016).

To counteract RNA silencing, viruses express viral suppressors of RNA silencing (VSRs), which inhibit various aspects of RNA silencing, including the binding of vsiRNAs or interfering with AGO protein function (Pumplin and Voinnet, 2013; Csorba et al., 2015). Genetic studies of RNA silencing in virus resistance can be difficult because compatible interactions between plant and virus necessarily mean that the virus is able to effectively inhibit the plant's defenses. The Potexvirus, *Plantago asiatica mosaic virus* (PlAMV) infects *Arabidopsis* (Yamaji et *al*., 2012) but moves systemically considerably slower than other *Arabidopsis*-infecting model RNA viruses, making it a good model for investigating increased susceptibility. Members of the Potexvirus family encode for a triple gene block (TGB) of three proteins that are involved in virus movement and establishing replication structures (Kim et *al*., 2014). The TGB1, or P25

protein of several Potexviruses has been shown to have VSR activity (Voinnet et al., 2000; Senshu et al., 2009; Lim et *al*., 2010). We have previously shown that PVX P25 destabilizes AGO proteins but also appears to protect the viral genome from the host cell machinery by creating viral replication complexes (Brosseau and Moffett, 2015). The P25 protein of *Plantago asiatica mosaic virus* (PlAMV) inhibits secondary vsiRNAs production by targeting the RDR6 pathway (Okano et *al*., 2014), however, the interaction between PlAMV and AGO proteins has not been reported.

We show an inverse correlation between destabilization of AGO proteins by PlAMV P25 and anti-viral activity against PlAMV and that the subset of AGO proteins involved in defense against PlAMV overlaps, but differs, from those involved in defense against PVX. Surprisingly, *ago4* mutant plants display the highest susceptibility to PlAMV and we present evidence suggesting that AGO4 can target PlAMV RNA. Furthermore, we show that PlAMV infection results in a change in localization of AGO4 from the nucleus to the cytoplasm, suggesting that the susceptibility of *ago4* mutant plants is most likely unrelated to the release of AGO4-mediated transcriptional silencing of defense genes. Together with our previous finding that AGO4 is required for resistance to TRV (Ma et al., 2015), this report further establishes a role for AGO4 in anti-viral defense.

3.3 Results

3.3.1 The *Arabidopsis* AGO4 protein curtails PlAMV infection

We tested a number of *Arabidopsis* lines mutated for genes known to be involved in defense, RNA silencing and DNA methylation for susceptibility to virus (Table 1; Supplementary Table S1). Mutant plants were inoculated with PlAMV expressing GFP (PlAMV-GFP) (Yamaji et *al*., 2012) and their relative susceptibility was assessed visually by the degree and speed of GFP spread throughout the plant, using a scale of one to six "+". Although subjective, this initial survey identified a number of mutants showing obviously increased or decreased susceptibility to PlAMV-GFP. Plants showing two or more or less "+" on the visual GFP scale, compared to

Table 1. Qualitative assessment of susceptibility to PlAMV of *Arabidopsis* mutants.

	Degree of susceptibility*		
Genotype	**5 dpi**	**10 dpi**	**15dpi**
Col-0 WT	++	++	+++
ago1–27	+	+	+
ago2–1	+++	+++	++++
ago3-2	+++	+++	++++
ago4-2	+++	+++++	++++++
ago4-5	+++	++++	+++++
ago5-1	++	++	+++
ago6-3	++	+++	++++
ago7	++	+++	++++
ago8-1	+++	+++	++++
ago8-2	++	++++	++++
ago9	++	++++	++++
ago10-2	++	+++	++++
ago1 ago2	+	++	++
ago1 ago2 ago10	+++	+++	nt
ago2 ago5 ago10	++	+++	++++
ago1 ago2 ago7	++	+++	++++
dcl2-1 dcl3-1 dcl4-2	++	+++++	++++++
dcl1-9	++	++	+++
dcl3-1	++	+++	+++
dcl4-2	++	++++	+++++
dcl2 dcl4	++	+++++	++++++
rdr1	++	+++	++++
rdr6	++	++	+++
nrpd1 nrpe1	++	nt	++++
nrpd1a-4	++	++	++++
nrpd1b-11	++	++	++++
nrpd2a	++	++	+++
Ler WT	++	++	+++
***ago4-1* (Ler)**	++	+++	+++++

*Susceptibility was determined by visualization, under UV illumination, of the extent of GFP accumulation and systemic spread in comparison with the appropriate WT background and (+) signs indicate relative degrees of susceptibility. nt, not tested. Gray shaded lines correspond to genotypes in which PlAMV accumulation is significantly different than in the WT.

Col-0 (for example, see Fig. 2A), were considered significant. This included the *bak1-4* mutant (Heese et *al*., 2011), a quadruple mutant deficient in four different defense-related hormone signaling pathways (Tsuda et *al*., 2009), as well as a *gh3.2* mutant, which is compromised in auxin signaling and is less susceptible to a necrotrophic fungus (Gonzalez-Lamothe et *al*., 2012) (Supplementary Table S1).

As expected, several RNA silencing mutants were more susceptible to PlAMV, including a triple dicer mutant (triple *dcl: dcl2 dcl3 dcl4*), which is largely deficient in anti-viral RNA silencing (Table 1; Fig. S1). Infection of individual *ago* mutant lines revealed some increased susceptibility in several lines (Table 1; Fig. S1). However, in contrast to most previous studies on the requirement for AGO proteins for defense against other RNA viruses, the *ago4-2* mutant showed the strongest increase in susceptibility at all time points, with a level of susceptibility to PlAMV similar to the *triple dicer* mutant (Table 1). To validate the involvement of individual AGO proteins using an alternate approach, we transiently overexpressed PlAMV-GFP with individual *Arabidopsis* AGO proteins in *N. benthamiana* leaves as previously reported with PVX (Brosseau and Moffett, 2015). As seen with PVX, overexpression of AGO2 and AGO5 resulted in lower levels of GFP from PlAMV, as visualized by UV illumination and immunoblotting (Fig. 1A and B). In addition, both AGO3 and AGO4 also restricted PlAMV-GFP accumulation in *N. benthamiana* leaves (Fig. 1A and B). The PlAMV P25 protein possesses a strong VSR activity in transient assays in *N. benthamiana* (Senshu et *al*., 2009). The P25 protein of PVX has been shown to destabilize certain AGO proteins (Chiu et *al*., 2010; Brosseau and Moffett, 2015) and we therefore monitored the accumulation of several *Arabidopsis* AGO proteins in the presence of PlAMV P25. Several AGO proteins that did not affect PlAMV accumulation in *N. benthamiana,* including AGO1, AGO6, AGO7, AGO9 and AGO10 showed lower levels of accumulation in the presence of PlAMV P25 (Fig. 1C). This result may explain why these AGO proteins have little or no effect on PlAMV accumulation when overexpressed in *N. benthamiana*. In contrast, AGO2, AGO3, AGO4 and AGO5 protein levels were unaffected by P25, suggesting that PlAMV is less able to overcome the antiviral activities of these proteins. Given that the AGO4 mutant was significantly more susceptible than

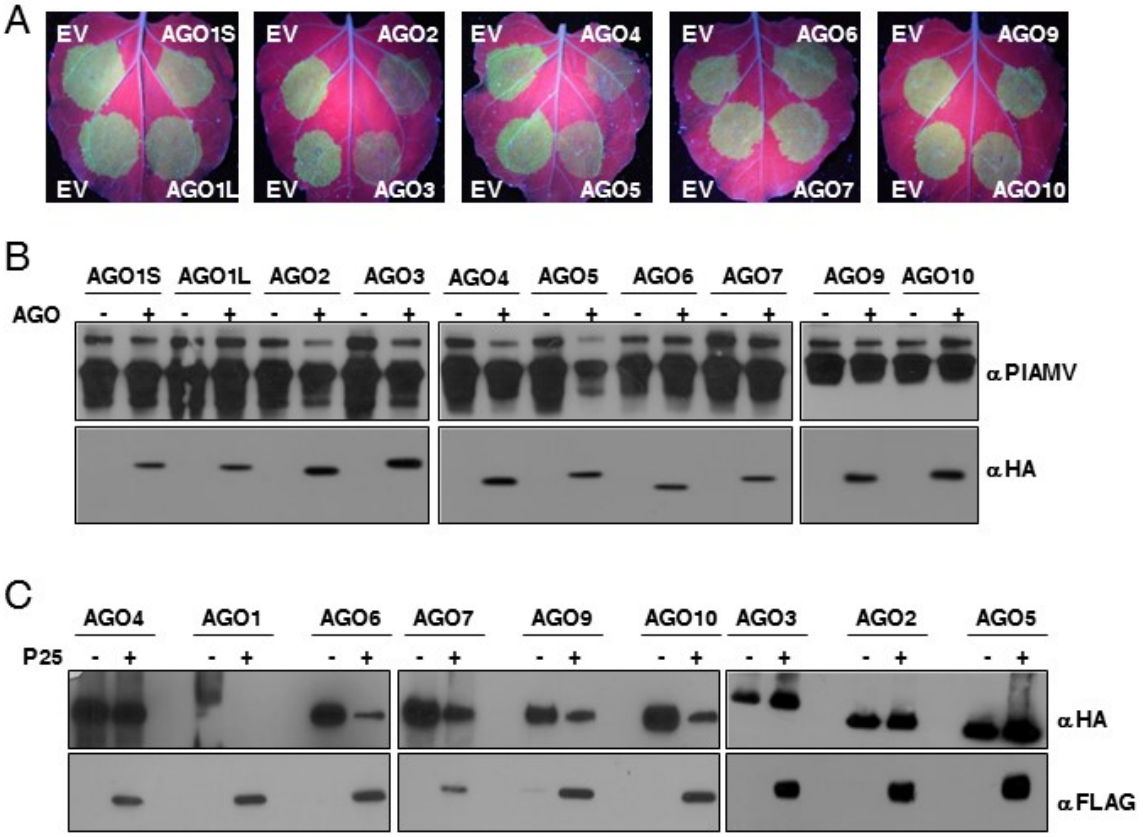

Fig. 1. Multiple *Arabidopsis* AGO proteins compromise PlAMV accumulation in *N. benthamiana*. **A,** *N. benthamiana* leaves were agroinfiltrated with PlAMV-GFP along with HA-tagged *Arabidopsis* AGO proteins or empty vector (EV), as indicated. Leaves were photographed under UV illumination 4 dpi. **B,** Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) 3 dpi and subjected to SDS-PAGE, followed by anti-PlAMV CP immunoblotting (top panel). (+) indicates that presence of the indicated AGO protein and (-) indicates EV. HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (lower panel). **C,** HA-tagged AGO proteins were coexpressed by agroinfiltration in *N. benthamiana* leaves with either FLAG-tagged P25 (+) or with empty vector (-). Total proteins were extracted and subjected to anti-FLAG immunoblotting (bottom panel). HA-tagged AGO proteins were immunoprecipitated and subjected to anti-HA immunoblotting (top panel). Experiments were performed three times and a representative result is shown.

all other AGO mutants tested, and the fact that AGO4 involvement in defense against RNA viruses has not been extensively studied, we focused further studies on AGO4 antiviral activity.

The *ago4-2* mutant allele contains a single missense mutation at amino acid 641. Although this mutation appears to result in a loss of function, it has also been reported to act as a dominant negative over the wild-type protein (Agorio and Vera, 2007), raising the possibility that susceptibility of this line to PlAMV may not result from absence of functional AGO4 but from non-functional AGO4 protein competing for siRNAs, substrates and/or partners. We thus infected an additional T-DNA knock-out *AGO4* mutant line, namely *ago4-5* (CS9927), with PlAMV and systemic spread of the virus was followed by UV visualization as well as protein and RNA analysis. PlAMV-derived GFP was detectable in systemic tissues of both *ago4* mutant lines at seven days post-inoculation (dpi), but not in Col-0 (Fig. 2A). However, there was a noticeable difference in resistance to PlAMV between these two mutants wherein *ago4-2* displayed higher susceptibility, manifested as visibly greater GFP accumulation, higher PlAMV coat protein (CP) and RNA accumulation in systemic leaves at both seven and nine dpi, as well as more severe symptoms at 21 dpi (Fig. 2A, B, C and D). A similar increased susceptibility to PlAMV was seen with the *ago4-1* null allele in the Ler background (Supplementary Fig. S2). As several AGO proteins appear to be implicated in restricting PlAMV, the increased susceptibility observed in *ago4-2* compared to *ago4-5* mutant may be caused by dominant negative function of this mutated AGO4 protein. Nevertheless, AGO4 also appears to be required *per se* to curtail PlAMV infection in both Col-0 and Ler backgrounds.

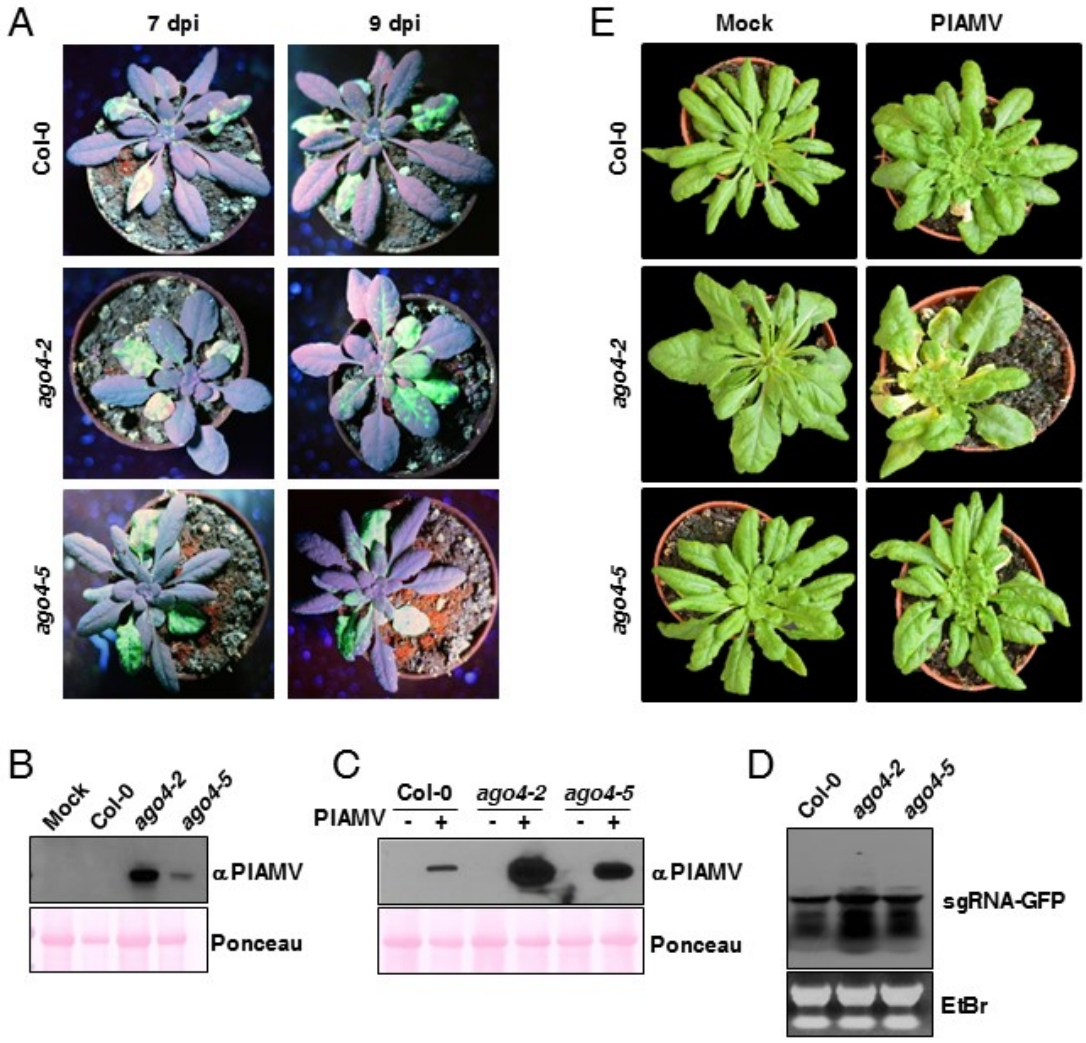

Fig. 2. *AGO4* knock-out lines display enhanced susceptibility to PlAMV. **A,** WT Col-0 *Arabidopsis* or *AGO4* mutant lines, as indicated, were inoculated with PlAMV-GFP. Plants were photographed under UV illumination at 7 dpi and 9 dpi. **B-C,** At 7 (**B**) and 9 (**C**) dpi, total protein extracts were prepared from systemic leaves and subjected to SDS-PAGE followed by anti-PlAMV CP immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Three plants per genotype were tested in each experiment and the experiment was repeated 5 times. **D,** At 8 dpi, total RNA was extracted from Col-0 WT, *ago4-2* or *ago4-5* mutant plants infected with PlAMV-GFP and subjected to northern blotting with an anti-GFP probe. PlAMV subgenomic (sg) RNAs are indicated. Ethidium

bromide (EtBr)- stained RNA is shown to demonstrate equal loading. The experiment was repeated three times and a representative result is shown. **E,** WT Col-0 *Arabidopsis* or *ago4* mutant lines, as indicated were either mock-inoculated or inoculated with PlAMV-GFP. Plants were photograph at 21 dpi.

3.3.2 PlAMV infection reduces AGO4 accumulation

Several genes encoding AGO proteins implicated in antiviral and antibacterial defense responses have been shown to be induced upon pathogen infection (Várallyay et *al*., 2010; Harvey et *al*., 2011; Zhang et *al*., 2011; Brosseau and Moffett, 2015; Wu *et al.*, 2015). We thus examined AGO4 protein levels upon PlAMV infection by immunoblotting. PlAMV infection resulted in a marked decrease in AGO4 protein accumulation in local inoculated leaves (Fig. 3A). AGO4 levels were also repressed in systemic leaves at eight dpi (Fig. 3A). At this time point, PlAMV is visible only in a relatively small portion of systemic leaves suggesting that this down-regulation may be non cell-autonomous.

Since the PlAMV P25 protein did not compromise the accumulation of AGO4 (Fig. 1C), we hypothesized that this regulation could be mediated at least in part at the transcriptional level. We therefore monitored *AGO4* expression by qRT-PCR analysis. Consistent with protein levels, *AGO4* transcripts were significantly less abundant after PlAMV infection in both local and systemic leaves (Fig. 3B).

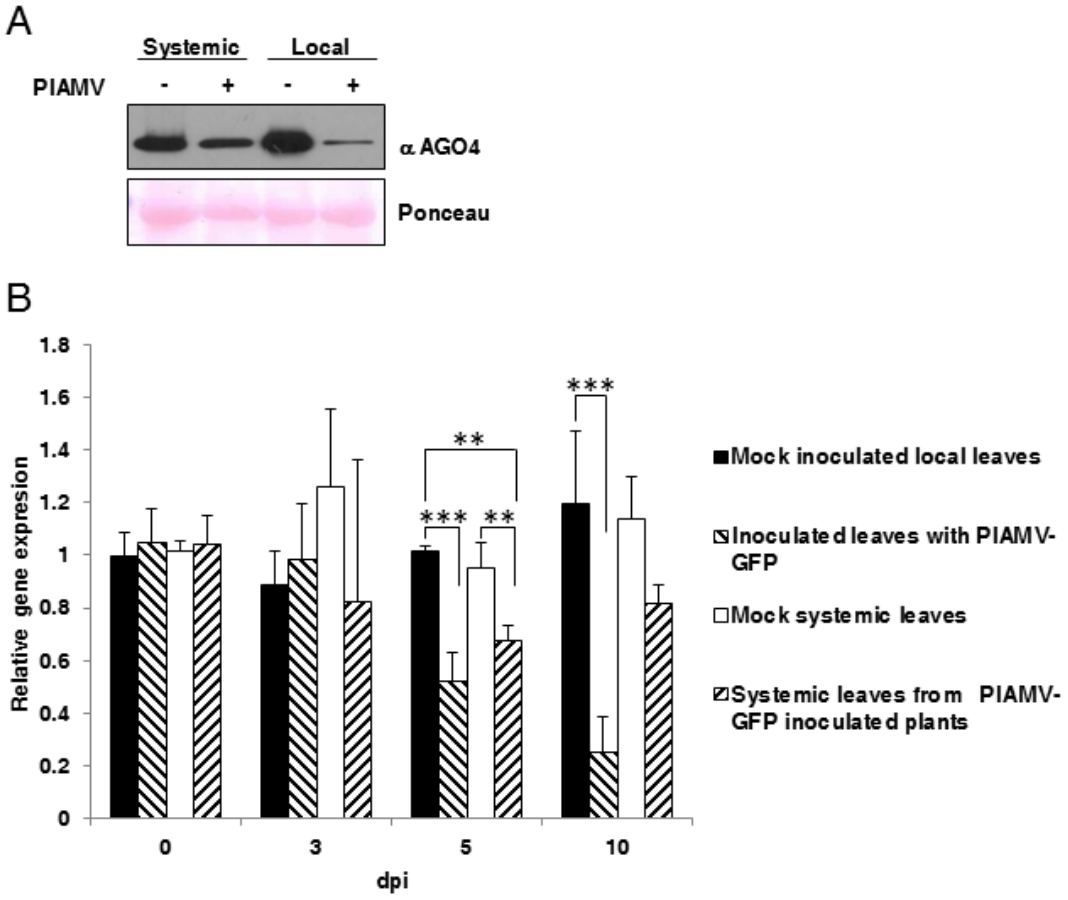

Fig. 3. PlAMV infection compromises AGO4 accumulation. Col-0 *Arabidopsis* were either mock inoculated or inoculated with PlAMV-GFP. **A,** At 8 dpi, total protein extracts were prepared from inoculated or systemic leaves and subjected to SDS-PAGE followed by anti-AGO4 immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Experiments were performed three times with similar results. **B,** Total RNA was extracted from inoculated and systemic leaves at indicated time points and subjected to qRT-PCR analysis to determine relative expression levels of AGO4. Relative gene expressions were analyzed using one-way ANOVA. Error bars represent standard deviation from three biological replicates. Data sets marked with asterisks are significantly different from WT plants at the same time point as assessed by Tukey's Studentized Range test (HSD): $***P < 0.01$ *or* $** P < 0.05$. The experiment was repeated at least three times with similar results.

3.3.3 Long term restriction of PlAMV requires mainly DCL2- and DCL4-derived siRNAs

AGO4 is known to preferentially bind 24-nt hcRNAs derived from the plant genome to form RdDM effector complexes (Mi et *al*, 2008; He et *al*., 2009; Havecker et *al.*, 2010; Gao et *al*., 2010; Wang et *al*., 2011). However, when expressed in the presence of a viroid, AGO4 binds mostly to 21- and 22-nt viroid-derived siRNAs (Minoia et *al*., 2015). Previous reports have demonstrated that both DCL2- and DCL4-produced sRNAs are required for optimal defense against ssRNA viruses (Deleris et *al*., 2006; Bouche et *al*., 2006; Diaz-Pendon et *al*., 2007; Dunoyer et *al*., 2007; Qu et *al*., 2008; Garcia-Ruiz et *al*., 2010; Andika et *al*., 2015; Brosseau and Moffett, 2015). However, AGO4 has been shown to predominantly bind heterochromatic siRNAs produced by DCL3 and to function in RNA-directed DNA methylation (RdDM) (Zilberman et *al*., 2003; Havecker et *al*., 2010). To investigate whether AGO4 antiviral activity is related to its function in endogenous gene regulation, we followed PlAMV infection in different *dcl* and *nrpd* mutants. At eight dpi, the *dcl1* mutation did not appear to result in accelerated movement of PlAMV into systemic leaves and at this time point, WT and *dcl1* mutants display similar accumulation of virus in systemic leaves as judged visually and by anti-PlAMV CP immunoblotting (Fig. 4A and B). Although single *dcl3* or *dcl4* mutants appeared slightly more susceptible than Col-0, they were nonetheless less susceptible than the *ago4-2* mutant. Indeed, only double *dcl2 dcl4* and triple *dcl* mutants were as susceptible as *ago4-2*, suggesting that optimal defense against PlAMV mainly depends on DCL2- and DCL4-derived sRNAs in early infection (Fig. 4A and B). However, at 21 dpi, mutation of *DCL4* alone appears to be sufficient to induce severe PlAMV symptoms whereas *DCL3* appears to be dispensable for optimal defense against PlAMV (Supplementary Fig. S3). Consistent with this observation, mutants of the *NRPD1a, NRPD1b* or *NRPD2a* genes, whose products encode subunits of the Pol IV and V complexes that are required for generating 24-nt hcRNAs, did not behave like *ago4* mutants as judged by PlAMV CP accumulation at nine dpi (Fig. 4C), although the *nrpd2a* mutant showed a slight increase in CP at nine dpi (Fig. 4C) and more severe symptoms at 21 dpi (Supplementary Fig. S4). Taken together, these results suggest that the antiviral function of AGO4 is independent of the canonical RdDM mechanism associated with AGO4-mediated epigenetic regulation, namely DCL3 and polymerases Pol IV and Pol V.

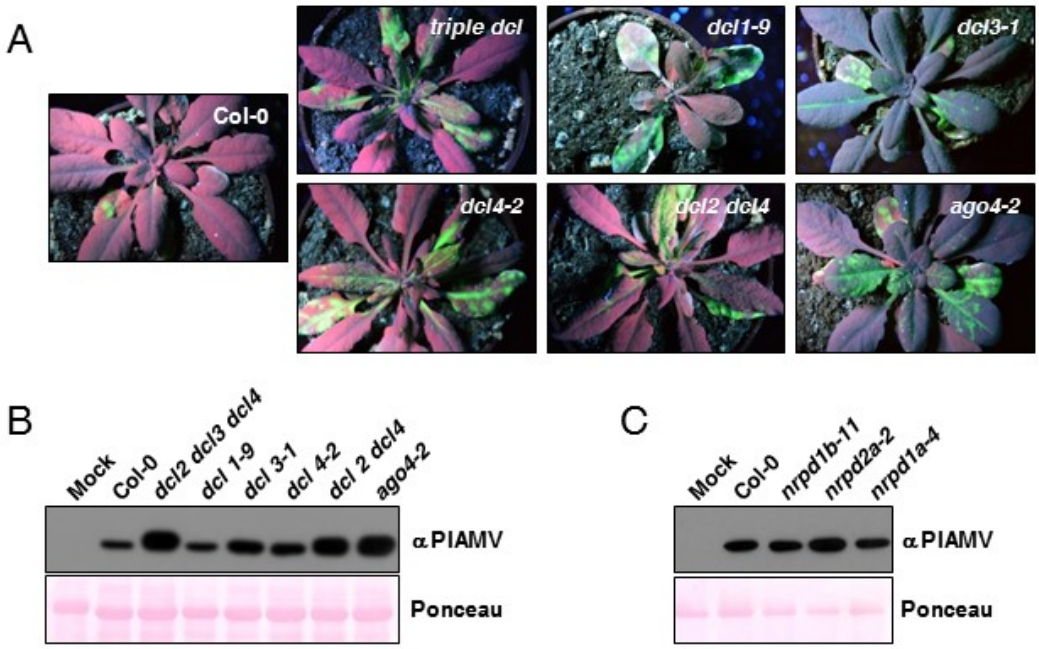

Fig. 4. Early defense against PlAMV requires DCL2 and DCL4 but not RdDM components. Col-0 WT *Arabidopsis* as well as single, double or triple *dcl* mutant lines, as indicated, were inoculated with PlAMV-GFP. **A,** Plants were photographed under UV illumination at 8 dpi. **B,** At 8 dpi, total protein extracts were prepared from systemic leaves and subjected to SDS-PAGE followed by anti-PlAMV immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. **C,** The indicated RdDM mutants were inoculated with PlAMV-GFP. At 9 dpi, total protein extracts were prepared from systemic leaves and subjected to SDS-PAGE followed by anti-PlAMV immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Three plants per genotype were tested in each experiment and the experiment was repeated four times.

3.3.4 AGO4 antiviral activity takes place in the cytoplasm

As canonical antiviral silencing mechanisms, but not RdDM, appear to be involved in the response to PlAMV, we hypothesized that AGO4 may function in the cytoplasm, the presumed location of PlAMV. To test this possibility, AGO4 was transiently expressed in *N. benthamiana* leaves in the presence or absence of PlAMV and the cytoplasmic/nuclear distribution of HA-tagged AGO4 was monitored by preparing cytoplasmic and nuclear fractions followed by anti-HA immunoblotting to detect HA-AGO4. As previously reported (Ye et *al*., 2012), in the absence of PlAMV, AGO4 is distributed in both the cytoplasm and the nucleus, but with a majority of the protein present in the nucleus (Fig. 5A). However, when co-expressed with PlAMV, AGO4 was still present in both cell compartments, but with a larger portion of the protein present in the cytoplasm (Fig. 5A). In agreement with the transient assays with P25, total AGO4 protein accumulation was not altered by co-expression with PlAMV (Fig. 1C). Transgenic *Arabidopsis* expressing AGO4 fused to eGFP expressed from the *AGO4* promoter (Ye et *al*., 2012) do not accumulate sufficient levels of GFP-AGO4 to allow visualization in leaves. However, this construct does allow sufficient accumulation in transient expression in *N. benthamiana* leaves to allow us to monitor cellular distribution by confocal microscopy. As previously reported, in the absence of virus, AGO4 showed a clear nuclear localization (Li et *al*., 2006; Pontes et *al*., 2006; Ye et *al*., 2012). However, consistent with the results obtained by biochemical fractionation, co-expression of PlAMV with GFP-AGO4 resulted in a shift towards a dispersed cytoplasmic localization of AGO4 (Fig. 5B).

The alteration of AGO4 localization could be due to sequestration by PlAMV or could be an active mechanism induced as a plant defense response. We thus transiently expressed PlAMV-GFP with WT AGO4 or a version of AGO4 lacking its nuclear localization signal (AGO4ΔNLS) expressed from the *AGO4* genomic promoter (Ye et *al*., 2012). In this assay, although AGO4ΔNLS was expressed beyond the limit of detection (Fig. 6B), both versions of AGO4 were equally efficient at reducing PlAMV-GFP accumulation (Fig. 6A and 6B). These results suggest that AGO4 antiviral activity is achieved through mechanisms similar to those used by

other AGO proteins and strongly suggest that the antiviral action of AGO4 is mediated in the cytoplasm.

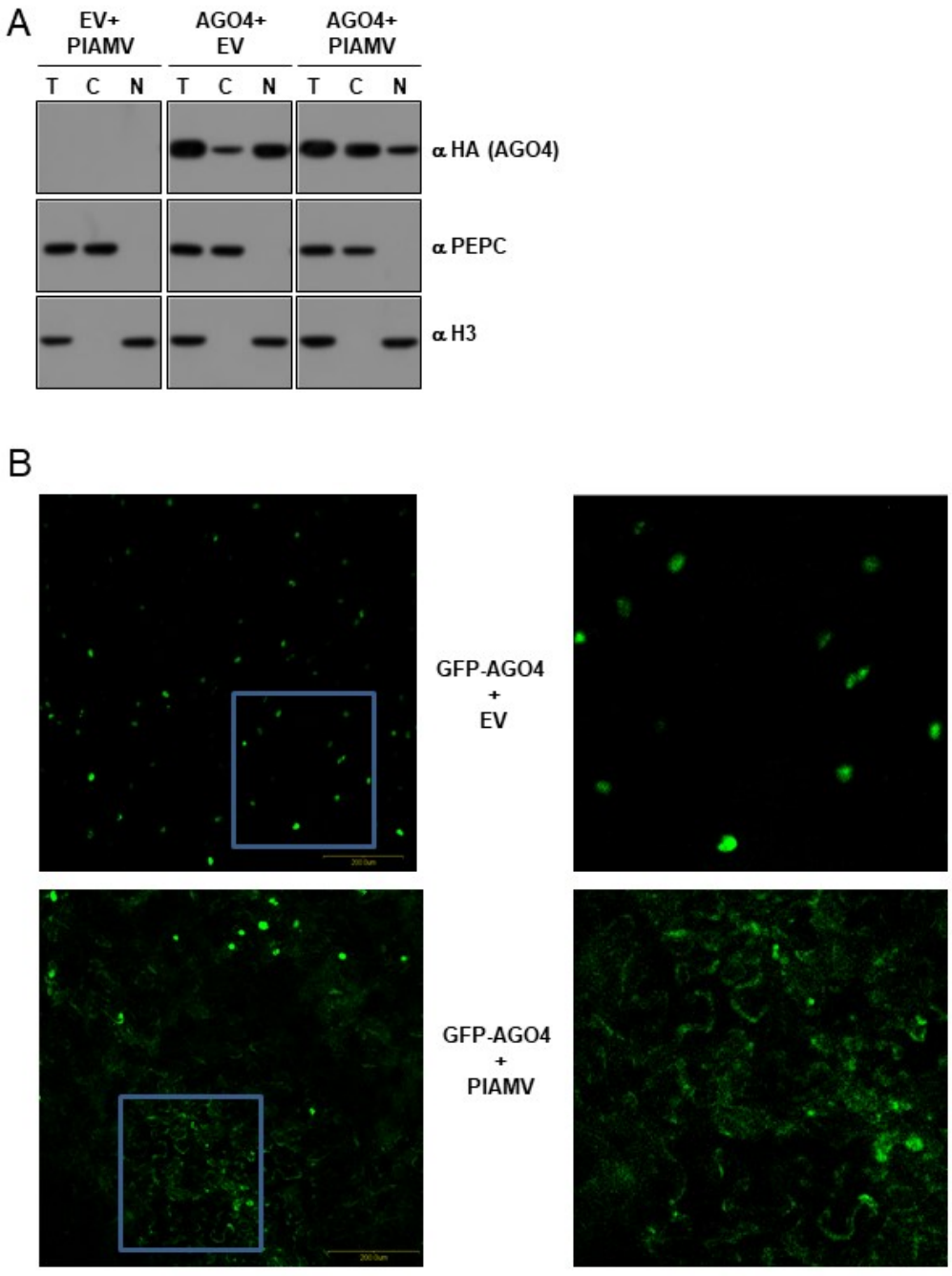

Fig. 5. A portion of the AGO4 pool is redistributed to the cytoplasm during PlAMV infection. **A,** HA-tagged AGO4 protein was coexpressed by agroinfiltration in *N. benthamiana*

leaves with either empty vector (EV) or PlAMV-GFP (PlAMV), as indicated. At 3 dpi, total protein extracts (T) were prepared as well as cytoplasmic (C) and nuclear (N) fractions. These fractions were then subjected to SDS-PAGE followed by anti-AGO4 immunoblotting (top panel). Anti-PEPC immunoblotting (middle panel) was used as protein marker for the cytoplasmic fraction, and anti-histone H3 immunoblotting (bottom panel) was used as a nuclear marker. **B,** P_{AGO4}:eGFP-AGO4 was coexpressed by agroinfiltration in *N. benthamiana* leaves with either EV or PlAMV, as indicated. At 3 dpi, eGFP-AGO4 fluorescence was observed by confocal microscopy. The boxed areas in the left panels are shown as close-ups in the right panels. The experiments were repeated at least three times with similar results.

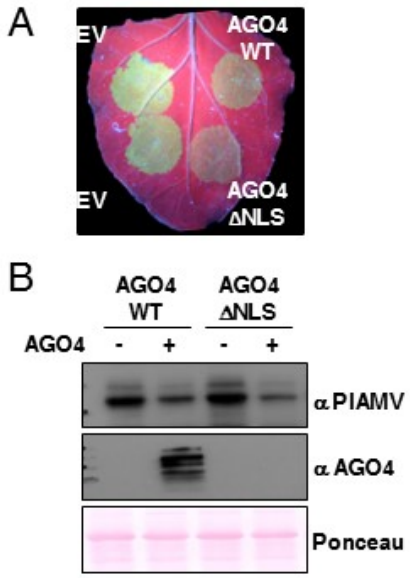

Fig. 6. AGO4 antiviral activity against PlAMV takes place in the cytoplasm. **A,** *N. benthamiana* leaves were agroinfiltrated with PlAMV-GFP along with either empty vector (EV) or P_{AGO4}:eGFP-AGO4 WT or P_{AGO4}:eGFP-AGO4ΔNLS. Leaves were photographed under UV illumination 4 dpi. **B,** At 4 dpi, total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in (A) and subjected to SDS-PAGE, followed by anti-PlAMV CP (top panel) or anti-AGO4 (middle panel) immunoblotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. The experiment was repeated at least three times with similar results.

3.4 Discussion

Previous studies have suggest that the AGO2 and AGO1 proteins play roles in defense against a range of RNA viruses (Carbonell and Carrington, 2015), although *ago1* mutants often show increased resistance against viruses such as TRV, TuMV, PVX (Ma et *al*., 2015; Garcia-Ruiz et *al*., 2015; Brosseau and Moffett, 2015) and PlAMV (Table 1). AGO4 has been shown to contribute to defense against plant DNA viruses by targeting their genomes for methylation (Raja et *al*., 2008; Raja et *al*., 2014). Through its predominant localization in the nucleus and its involvement in transcriptional gene silencing (TGS) and RdDM (Zilberman et *al*., 2003; Li et *al*., 2006; Wierzbicki et *al*., 2009; Gao et al., 2010; Greenberg et *al*., 2011; Ye et *al*., 2012), the antiviral action of AGO4 against DNA viruses is intuitive. However, an involvement of AGO4 in counteracting RNA viruses has not been well described. Although VSRs, such as CMV 2b have been shown to inhibit AGO4 activity and the TRV 16K protein binds to AGO4 (Hamera et *al*., 2012; Fernandez-Calvino et *al*., 2016), the significance of these results is not clear. VSR targets may not always be specific due to mechanisms that broadly target RNA silencing components (i.e. broadly targeting most/all AGO proteins or sRNAs). Indeed, increased susceptibility of *ago4* mutants has only been reported for one RNA virus, TRV (Ma et *al*., 2015; Fernandez-Calvino et *al*., 2016). AGO4 has been previously shown to be required for defense against biotrophic pathogens, as well as for NB-LRR mediated defenses, presumably through its role in gene regulation (Bhattacharjee et *al*., 2009; Agorio and Vera 2007; Hamera et *al*., 2012; Dowen et *al*., 2012). Although, as suggested by Hamera et *al*., (2012), it is possible that AGO4 is involved in the transcriptional response to virus infection, our study, combined with recent reports suggest an alternate mechanism for AGO4 antiviral activity.

3.4.1 AGO4 possesses antiviral function independent of the RdDM pathway

Several studies have shown widespread dynamic differential methylation in response to pathogen infection and it has been suggested that altered susceptibility to non-viral pathogens in *ago4* mutants may be due to the role of AGO4 in DNA methylation (Lopez et *al*., 2011; Dowen et *al*., 2012; Yu et *al*., 2013; Hamera et *al*., 2012). However, in the case of PlAMV, the

defense response is most likely not entirely at the transcriptional level. First, genetic analysis indicates that DCL3 does not play a major role in resistance to PlAMV (Fig 4A and B; Supplementary Fig. S3). This suggests that AGO4 antiviral activity does not involve the canonical TGS activity mediated by DCL3-produced 24-nt sRNAs, although we cannot rule out the possibility of TGS mediated through association of AGO4 with 21- or 22-nt sRNA. Secondly, both nuclear/cytoplasmic fractionation and microscopy analysis showed that AGO4 is predominantly localized in the cytoplasm upon PlAMV infection (Fig. 5). These results suggest that AGO4 acts against viruses in the cytoplasm rather than through RdDM, which takes place in the nucleus (Li et *al*., 2006; Wierzbicki et *al*., 2009; Gao et *al*., 2010). Although the remaining AGO4 protein present in the nucleus could be sufficient to induce transcriptional changes to restrict PlAMV, we favor a cytoplasmic mode of action because expression of AGO4ΔNLS also restricts PlAMV (Fig. 6). Furthermore, the observation that mutation of different genes in the RdDM pathway does not lead to the same increase in susceptibility as the *ago4* mutants indicates that AGO4 antiviral function against PlAMV is at least partially independent of RdDM (Table 1, Fig. 4C; Supplementary Fig. S4).

Plants deficient in SA signaling (*pad4*) are more susceptible to PlAMV, while plants constitutively activated in SA signaling (*snc1*) are more resistant (Supplementary Table S1), consistent with studies showing an involvement of SA in defense against viruses (Chivasa et *al*., 1997; Naylor et *al*., 1998; Murphy et *al*., 1999; Lee et *al*., 2011). Whether AGO4, SA and BAK1-mediated defenses are related remains to be seen, although if AGO4 were a downstream component of PTI or SA signaling it would be expected to be required for resistance against other viruses. However, whereas plants deficient in SA are more susceptible to VSR-deficient CMV, *ago4* mutants are not (Shang et *al*., 2011; Wang et *al*., 2011b). Likewise, *bak1*, but not *ago4*, mutants show increased susceptibility to TCV (Kørner et *al*., 2013; Zhang et al, 2012). Thus, we suggest that the specificity in requirement for AGO4 is consistent with the differential requirement for other AGO proteins reported for other plant-virus interactions. That is, different AGO proteins contribute to virus resistance depending on the ability of the virus to counteract their activity, as well as their ability to access viral RNA.

3.4.2 AGO4 expression and localization

The increased presence of AGO4 in the cytoplasm versus the nucleus in the presence of PlAMV could be due either to a direct effect of the virus on AGO4 or an indirect effect. A previous study has shown that the localization of the RNA silencing component SGS3 is altered by the PlAMV VSR, P25 (Okano et *al*., 2014). In this case P25 appears to enwrap SGS3 bodies in localized cytoplasmic foci and curtails their ability to generate secondary siRNAs. However, AGO4 does not appear to localize to specific foci in the presence of PlAMV (Fig. 5) and is thus not likely affected in the same way as SGS3. Likewise, AGO4 function does not appear to be compromised by PlAMV, as AGO4 co-expression with PlAMV restricts virus accumulation and AGO4 stability is not affected by P25 (Figs. 1 and 5A). Thus the effect of PlAMV on AGO4 localization may be indirect. This effect is not likely due to AGO4 overexpression as GFP-AGO4 localization in our experiments (Fig. 5B) appears to be the same as when it is expressed from the AGO4 promoter in transgenic plants (Ye et *al*., 2012). The nature of this effect will require further study as the nuclear localization of AGO4 is affected by several factors, including cytoplasmic loading of siRNAs, cleavage activity and Hsp90 function (Ye et *al*., 2012). The increased cytoplasmic localization of AGO4 could be due to an inhibition of AGO4 loading of 24-nt sRNAs. However, this seems unlikely as PlAMV P25 transgenic plants show compromised accumulation of 21-nt, most likely secondary siRNA, but not 24-nt, sRNAs (Okano et *al*., 2014). Alternatively, the movement of AGO4 out of the nucleus may be an active defense response on the part of the plant. In this scenario, we hypothesize that the plant cell responds to the presence of virus and localizes AGO4 to the cytoplasm where it directly targets viral RNA. Although we do not see a difference in overall AGO4 accumulation in the presence of PlAMV (Fig. 1B, 5A), the GFP-AGO4ΔNLS protein was not detectable, suggesting that its inability to transit to the nucleus may result in low levels of accumulation. However most plant AGO proteins intrinsically accumulate to very low levels, requiring concentration by immunoprecipitation to detect by immunoblotting (see materials and methods) and GFP-AGO4ΔNLS still had a clear effect on PlAMV (Fig. 6).

Interestingly, our results demonstrate that, even if AGO4 is required to restrict PlAMV, its expression decreases during the course of infection (Fig. 3). This down-regulation does not appear to be age-related as mock infected plants do not show a similar decrease over time (Fig. 3B). This is consistent with a previous study showing that *AGO4* is downregulated in *N. benthamiana* in response to PVX infection (Ye et *al*., 2009). *AGO4* downregulation does not appear to be virus-specific as it is also downregulated in response to flagellin detection and infection by *Pseudomonas syringae* (Yu et *al*., 2013). Whether this phenomenon is induced by the pathogen to increase susceptibility or if it is a decision of the plant to shift toward other defense pathways, is not clear. It is interesting to note however, that both the induction of *AGO5* expression (Brosseau and Moffett, 2015) and the downregulation of *AGO4* (Fig. 3) occur in systemic leaves before widespread infection of PlAMV in these tissues. For example, AGO4 protein levels are decreased in systemic leaves at 5 dpi (Fig. 3A), whereas PlAMV-GFP is still not visibly detectable in systemic leaves at 7 or 8 dpi (Figs. 2A, 4A). Indeed, when transiently expressed from a constitutive promoter, AGO4 protein levels are not affected by the presence of PlAMV. This suggests that AGO4 levels may be affected by PlAMV infection at the transcriptional or post transcriptional level and that a systemic signal may be involved.

3.4.3 Virus defense and counter defense

A direct action of AGO4 on virus resistance may seem counterintuitive as it is best characterized for its role in epigenetic regulation (Matzke et *al*., 2015). However, we have shown that all *Arabidopsis* AGO proteins have the intrinsic ability to target PVX lacking its triple gene block proteins, presumably because the viral RNAs are not protected either by a VSR or by viral replication complexes induced by these proteins (Brosseau and Moffett, 2015). Likewise, most AGO proteins, including AGO4, can bind to 21- and 24-nt sRNAs derived from viroids and overexpression of AGO1, 2, 4 and 5 attenuates viroid accumulation (Minoia et *al*., 2014). This has lead us to suggest that the requirement for different AGO proteins for defense against viruses may depend on the properties or replication strategies of the virus in question (Ma et *al*., 2015; Brosseau and Moffett, 2015). To be able to target a virus, a given AGO protein must not be inhibited by a VSR, but must also be able to access viral RNA. In agreement with this, only

those AGO proteins not destabilized by the P25 show activity against PlAMV (Fig. 1). Mutants of those AGO proteins that do target PlAMV in transient assays do not all show phenotypes however, probably because the strong activity of AGO4 masks their contribution. We note that the number of AGO proteins targeting PVX (AGO2 and AGO5) is less than PlAMV (AGOs 2, 3, 4 and 5) and speculate that this is due to a combination of VSR targeting specificity and RNA accessibility, the latter due to differences in viral replication complexes. The increased susceptibility of PlAMV to AGO proteins however, may be less important given that its P25 strongly suppresses the production of secondary siRNAs by the SGS3/RDR6 pathway whereas PVX P25 does not (Okano et *al*., 2014).

This report further underlines the importance of using both functional and genetic approaches to understanding anti-viral RNA silencing and solidifies the involvement of AGO4 in resistance to at least some viruses. In addition, our observation of the alteration of AGO4 localization suggests both an explanation for how this presumed nuclear protein functions in combating viruses in the cytoplasm as well as paving the way for future work aimed at understanding this phenomenon.

3.5 Materials and Methods

3.5.1 Plant material and growth conditions

Nicotiana benthamiana and *Arabidopsis thaliana* plants were grown in BM6 (Berger) and Agromix (Fafard), respectively, in growth chambers with 16-h-light/8-h-dark and 12-h-light/8-h-dark photoperiod at 23°C and 21°C respectively. Except for the *ago4-5* mutant line (ABRC CS9927), all *Arabidopsis* mutant lines have been described previously, including the *ago1-27* (Morel et *al*., 2002), *ago2-1*, *ago3-1*, *ago6-3*, *ago8-1*, *ago10-2* (Takeda et *al*., 2008), *ago5-1*, *ago7, ago9* (Katiyar-Agarw*al* et *al*., 2007), *ago4-1* (Zilberman et al., 2003), *ago4-2* (Agorio and Vera, 2007), *ago1 ago2*, *ago1 ago2 ago7*, *ago2 ago7, ago2 ago5 ago10, ago1 ago2 ago10* (Wang et *al.*, 2011b), *triple dicer* (Deleris et *al*., 2006), *dcl1-9* (Jacobsen et *al*., 1999), *dcl2-1* and *dcl3-1* (Xie et *al*., 2004) and *dcl4-2* and *dcl2 dcl4* (Xie et *al*., 2005), *rdr1-1* (Xie et *al*.,

2004), *rdr6-15* (Boccara et *al*., 2014), *nrpd2a* (Lopez et *al*., 2011), *nrpd1a-4*, *nrpd1b-11* and *nrpd1 nrpe1* (Pontier et *al*., 2005).

3.5.2 Plasmid construction

For the generation of pBIN61-P25-FLAG, the P25 CDS was amplified with following primers 5'-tctagaATGGACATAGTCATCTCAGC-3' and 5'- ggatccGTAGGTGGGGTGAG-3' using PlAMV-GFP (Yamaji et *al*., 2012) as template. All other constructions have been previously described including pBIC-HA-AtAGO constructs (Takeda et *al*., 2008), PlAMV-GFP (Yamaji et *al*., 2012), PlAMV (Ozeki et *al*., 2006), pAGO4:GFP-AGO4 and its ΔNLS variant (Ye et *al*., 2012).

3.5.3 Virus inoculation in *A. thaliana*

Infections of three-week-old *Arabidopsis* plants were carried out by rub inoculation as previously described (Brosseau and Moffett, 2015) except that the inoculum was diluted 1:4 in phosphate buffer (i.e. 8 ml/g of infected tissues).

3.5.4 Transient expression in *N. benthamiana*

Agrobacterium-mediated transient expression assays in *N. benthamiana* were performed as previously described (Brosseau and Moffett, 2015).

3.5.5 RNA analysis

Northern blotting was performed as previously described using a GFP probe (Ma et *al*., 2015). For RT-qPCR analysis, total RNA was extracted from leaf samples using the RNeasy Plant mini kit according to the manufacturer's recommendation (Qiagen, Maryland, USA). RNAs were treated with RNAase free DNase (Qiagen, Maryland, USA). First-strand cDNA was synthesized

from 2 µg total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The *AGO4* gene was amplified using following primers AtAGO4F: TGA GGC ATT ACC ACC TCC TC and AtAGO4R: CGA GCC ATA GGA ACT CGA AC. The qPCR was performed as previously described (El Oirdi et *al*., 2011; Gonzalez-Lamothe et *al*., 2012) with some modifications, using the Eva Green method according to the manufacturer's recommendation (BioRad). Melting curves were determined using the dissociation curve software operated by CFX Manager™ software (version 3.0) to ensure that only a single product was amplified. The Biorad CFX96™ real-time PCR system (Bio-Rad) operated by CFX Manager™ software (version 3.0) was used to detect the amplification level and was programmed with an initial step of 98°C for 2 min and 40 cycles of 98°C for 2 sec and annealing /extension at 60°C for 5 sec with melt curve analyses is from 65 to 95°C in 0.5°C increments. All reactions were run in technical triplicates for each biological replicate, and the average values were used for quantification. The relative quantification of target genes was determined using the CT method. Briefly, the Ct (threshold cycle) values of target genes were normalized to an endogenous control gene (CT = Ct target – Ct endogenous) and compared with a calibrator (CT = Ct sample – Ct calibrator). Relative expression (RQ) was calculated using the BioRad sequence detection system software and the formula RQ = 2-CT.

AtEF1 was used as an endogenous control for plant target by using following Primers AtEF1α F: TCTCCGAGTACCCACCTTTG and AtEF1α R: TCCTTCTTGTCCACGCTCTT.

3.5.6 Statistical Analysis

Relative gene expressions were analyzed using one-way ANOVA. Error bars represent standard deviation from three biological replicates. Data sets marked with asterisks are significantly different from WT plants at the same time point as assessed by Tukey's Studentized Range test (HSD): ***$P < 0.01$ or **$P < 0.05$. The experiment was repeated at least three times with similar results.

3.5.7 Protein extraction and analysis

For AGO expression analysis, 1 g of fresh tissue was ground into 2 mL of RISC buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 0.5% NP-40) supplemented with protease inhibitor cocktail. Total protein extracts were centrifuged at 16 000 xg for 10 minutes at 4°C. A fraction of total protein extract was kept for detection of PlAMV. Immunoprecipitation was carried out with 1.4 ml of supernatant and 25 µl of HA-agarose beads (Sigma) for 2 h at 4°C on a rotatory shaker. Beads were washed 4 times with RISC buffer. After centrifugation, beads were resuspended in 50 µl of 1.5X of Laemmli loading buffer. Proteins were separated by SDS-PAGE on 7.5% or 10.5% acrylamide gels for AGO or GFP and CP detection respectively, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by electroblotting. HA-AGO proteins were probed with anti-HA-horseradish peroxidase conjugated (HRP) antibodies (Sigma, 1:3,000 dilution). Detection of GFP was carried out by probing membranes with anti-GFP-HRP antibodies (Santa Cruz, 1:3,000 dilution). Anti-PlAMV-CP rabbit polyclonal antibodies (Agdia, 1:20,000 dilution) were used to detect PlAMV, followed by donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend, 1:10,000 dilution). Detection of *Arabidopsis* AGO4 was carried out by probing membranes with anti-AGO4 antibody (Agrisera, 1:4000 dilution) and subsequently with donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend, 1:10,000 dilution).

3.5.8 Nuclear-cytoplasmic fractionation

Nuclear/cytoplasmic fractionation was performed as previously described (Wang et *al*., 2011c) with minor modifications. AGO4 present in cytoplasmic fraction was concentrated by IP. The purified nuclear pellets were homogenized in Trizol to extract proteins as per the manufacturer's instructions (Ambion).

3.5.9 Confocal microscopy

Three days after agroinfiltration, leaf discs from infiltrated patches were mounted between slide and coverslip and GFP-AGO4 fluorescence was monitored with a 20X objective, using a 488nm argon laser. Emitted fluorescence was collected using a 510-530 band-pass filter and a 575-630 band-pass on an Olympus FV300 confocal microscope.

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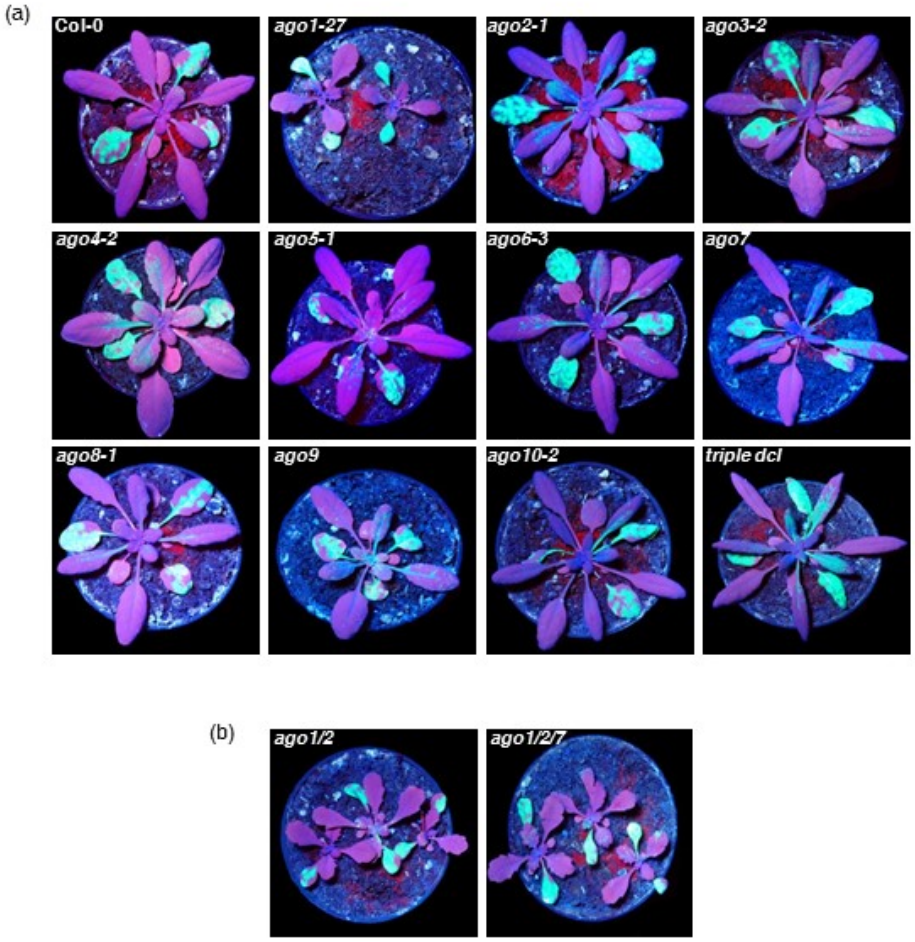
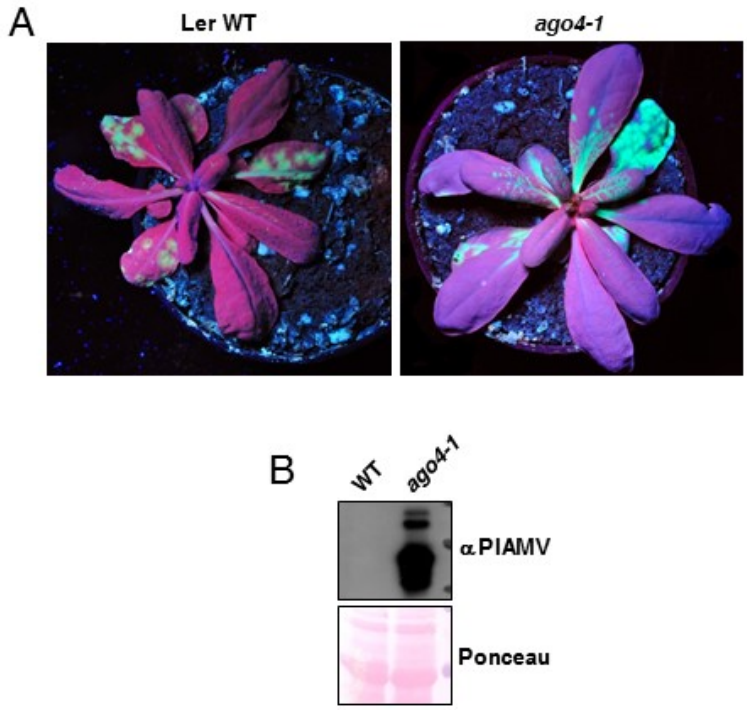
3.8 Supplemental data

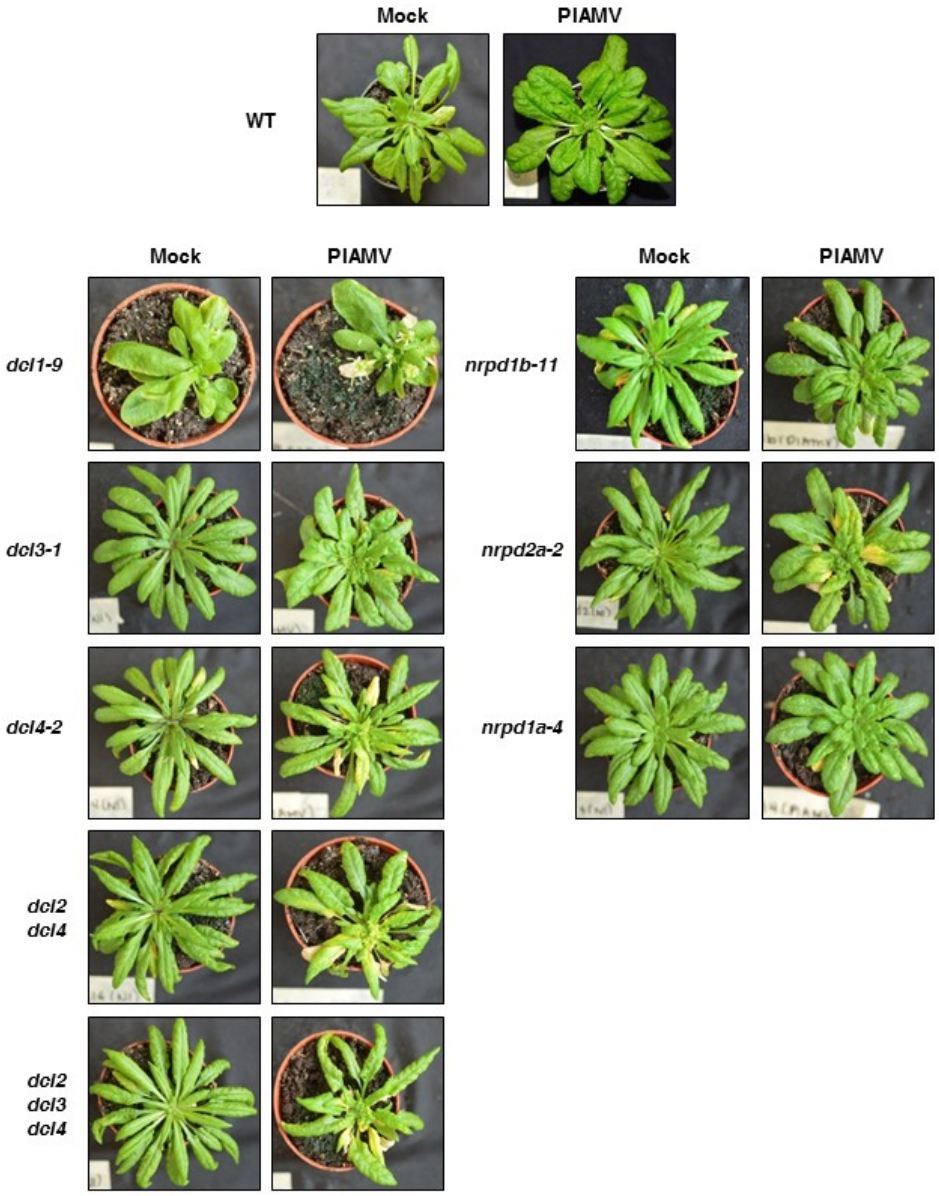
3.8.1 Supplemental Figures



Supplemental Fig. S1. Multiple *Arabidopsis* AGO proteins cooperate to curtail PlAMV infection. A-B, Col-0 WT *Arabidopsis* as well as single (A), double and triple ago (B) mutant lines, as indicated, were inoculated with PlAMV-GFP. Plants were photographed under UV illumination 8 days post inoculation (dpi). At least three plants per genotype were tested in each experiment and the experiment was repeated at least three times for each genotype.



Supplemental Fig. S2. *ago4* mutation in the Ler background increase susceptibility to PlAMV. A, WT Ler *Arabidopsis* and the *ago4-1* mutant, as indicated, were inoculated with PlAMV-GFP. Plants were photographed under UV illumination at 8 dpi. **B,** At 8 dpi, total protein extracts were prepared from systemic leaves and subjected to SDS-PAGE, followed by anti-PlAMV CP immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Three plants per genotype were tested in each experiment and the experiment was repeated five times.



Supplemental Fig. S3. Enhanced PlAMV symptom severity on *dcl2 dcl4* and *nrpd2a* mutants. WT Col-0 *Arabidopsis*, double or triple *dcl* mutant lines, as well as mutants in Pol IV (*NRPD1A* and *NRPD2A*) and Pol V (*NRPD1A* and *NRPD1B*), as indicated, were either mock inoculated or inoculated with PlAMV-GFP. Plants were photographed at 21 dpi. Three plants per genotype were tested in each experiment and the experiment was repeated at least three times.

3.8.2 Supplemental table

Supplemental Table S1. Relative susceptibility to PlAMV of different *Arabidopsis* mutants.

		Degree of susceptibility*		
	Seed stock/Reference	**5 dpi**	**10 dpi**	**15dpi**
Col-0 WT	CS28168	++	++	+++
dde2-2 ein2-1 pad4-1 sid2-2	N66007	++	+++	+++++
sid2-2	CS16438	++	++	++++
dde2-2	CS65993	++	++	++++
pad4-1	CS3806	+++	++++	nt
mir472	SALK_087945C	++	+++	++++
fls2	SALK_062054	+	++	++++
bak1-3	SALK_034523	+++	++++	nt
bak1-4	SALK_116202	++++	+++++	nt
snc1	Li et *al*., 2001	+	+	nt
gh3-2	Gonzalez-Lamothe et *al*., 2012	++	++++	nt

*Susceptibility was determined by visualization, under UV illumination, of the extent of GFP accumulation and systemic spread in comparison with the appropriate WT background and (+) signs indicate relative degrees of susceptibility. nt, not tested. Gray shaded lines correspond to genotypes in which PlAMV accumulation is significantly different than in the WT.

CHAPITRE 4

LA VARIABILITÉ GÉNÉTIQUE DANS LE DOMAINE N-TERMINAL D'AGO2 CHEZ *ARABIDOPSIS* MODULE L'ACTIVITÉ ANTIVIRALE DE LA PROTÉINE.

Dans cet article, nous avons étudié comment la variabilité retrouvée dans la séquence codante d'*AGO2* module l'activité antivirale de la protéine. D'abord, nous avons observé une grande variabilité, entre différents écotypes d'*Arabidopsis thaliana*, au niveau de la séquence codant pour AGO2. Par des essais d'infection chez différents écotypes d'*Arabidopsis* et par des essais transitoires chez *N. benthamiana*, nous démontrons que certains polymorphismes retrouvés dans le domaine N-terminal affectent négativement l'activité antivirale. De façon intéressante, nous avons observé que ces polymorphismes n'affectent pas les fonctions anti-bactérienne et de méthylation de l'ADN de la protéine AGO2. La grande majorité des études à ce jour se sont concentrées à caractériser les domaines fonctionnels retrouvés dans la partie C-terminal des protéines AGO. Ces résultats fournissent des pistes d'étude sur l'importance et la fonction du domaine N-terminal dans les interactions plante-virus. Cette première étude réalisée chez une plante modèle ouvrira la voie à d'autres études qui seront réalisées chez des plantes d'intérêt agronomiques afin de trouver des cultivars ayant une AGO2 plus fonctionnelle au niveau de la défense antivirale.

Pour cet article CB, AA et PM ont conçu les expériences. CB a réalisé la grande majorité des expériences. AA a réalisé la caractérisation des RILs par PCR ainsi que les infections bactériennes avec *Pseudomonas*. SB a effectué trois réplicas des essais transitoires avec NbAGO2. CRL a réalisé deux réplicas pour les infections virales des RILs et les qPCRs pour analyser l'expression relative d'AGO2 (Fig. 6c). ZZ a réalisé la figure supplémentaire 5. CB et PM ont rédigé le manuscrit. Le manuscrit sera soumis à la revue scientifique Nature plants.

4. Natural variation in the *Arabidopsis AGO2* gene is associated with susceptibility to PVX

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4.1 Abstract

RNA silencing functions as an anti-viral defence through the action of DICER-like (DCL) and ARGONAUTE (AGO) proteins. We have previously shown that the AGO2 protein possesses an important antiviral activity in both *Arabidopsis thaliana* (At) and *Nicotiana benthamiana* (Nb) against the Potato virus X (PVX) and Tomato bushy stunt virus (TBSV), respectively. Given the fact that NbAGO2 and AtAGO2 proteins share low sequence identity, it was difficult to identify specific residues important for anti-PVX defense. Here, using natural genetic diversity between the *Arabidopsis thaliana* accessions, we found two polymorphisms in N-terminus of AGO2 sequence that specifically affect its antiviral activity without interfering with other AGO2 functions. Moreover, we show that introgression of a Col-0-like-AGO2 into a PVX-susceptible ecotype confers full resistance. Our results indicate that natural variation in RNA silencing components may be an avenue for improving resistance to pathogens.

4.2 Introduction

RNA silencing is a conserved gene regulatory mechanism that is also by plants to counteract virus infection(Carbonell and Carrington, 2015). Virus double-stranded RNA (dsRNAs), produced during the replication of RNA viruses, is recognized and cleaved into small-interfering RNAs (siRNAs) by dicer-like (DCL) proteins. These siRNAs are then incorporated into an RNA-silencing complex (RISC) which contains Argonaute (AGO) endoribonucelase proteins. These programmed complexes then target any single-stranded (ssRNA) with sufficient complementarity for cleavage or translation inhibition(Omarov et al., 2016).

To counteract RNA silencing, plant viruses encoded viral suppressors of RNA silencing (VSR), which have been shown to interfere with multiple different steps of this mechanism(Pumplin and Voinnet, 2013). The potato virus X (PVX) VSR is the P25 protein([CSL STYLE ERROR: reference with no printed form.])·(Chiu et al., 2010), also known as 25K or TGB1, which is also implicated in viral movement and in the formation of PVX viral replication complex (VRC).

Among the 10 AGO proteins encoded by Arabidopsis thaliana, AGO2 has been identified most often as having antiviral function and this, against different viruses(Carbonell and Carrington, 2015). An antiviral role for AGO2 appears to be conserved in *Nicotiana benthamiana* (Scholthof et al., 2011)·(Odokonyero et al., 2015)·(Fátyol et al., 2016)·(Ludman et al., 2017). However, despite the presence of an antiviral AGO2, *N. benthamiana* is susceptible to a wide range of viruses, including PVX(Bally et al., 2015). Although different studies identified important functional amino acid in AGO proteins(Fátyol et al., 2016)·(Poulsen et al., 2013), little is known on how an AGO2 can or cannot target a potexvirus.

Here, we have shown, using transient expression assays, that AGO proteins from *A. thaliana* and *N. benthamiana* show differing activities against PVX, suggesting that inter-specific differences in AGO2 may contribute to differing outcomes in PVX infection in these species. To test whether intra-specific differences in AGO2 might relevant to plant-virus interactions we have taken advantage of natural genetic variation of wild *Arabidopsis thaliana* accessions (CS76427). We show the *AGO2* gene presents a high level of polymorphism and that, unlike the commonly used *A. thaliana* accession Col-0, 27 out of 63 accessions analysed are susceptible to PVX. This susceptibility is determined by two polymorphisms found in the N-terminus of the AGO2 protein, suggesting that natural variation in AGO2 may be important for determining plant-virus interaction outcomes.

4.3 Results

4.3.1 AGO2 proteins from different genera display specific antiviral activity

To determine if differences in PVX susceptibility between *N. benthamiana* and *A. thaliana* might be determined in part by AGO2, we transiently expressed the two proteins with PVX-GFP. Consistent with our previous results(Brosseau and Moffett, 2015), transient expression of AtAGO2 in *N. benthamiana* resulted in a lower PVX-derived GFP accumulation (Fig. 1a, 1b). However, NbAGO2 had much less effect on PVX accumulation, as determined visually and by immunoblotting (Fig. 1a, 1b). Despite being expressed under the same strong promoter, the two

AGO2 proteins did not accumulate at similar levels in this experiment (Fig. 1b, middle panel). Previous studies have shown that the Potexvirus VSR, P25, has the potential to compromises stability or accumulation of different AGO proteins(Brosseau and Moffett, 2015; Brosseau et al., 2016; Chiu et al., 2010). To monitor whether the presence of P25 affects NbAGO2 antiviral activity, we used a mutated version of PVX, PVX-GFPΔTGB, which lacks components required for suppression of silencing (P25) as well as for the formation of the PVX X-bodies and for cell-to-cell movement. In contrast to WT PVX, both AGO2 proteins significantly reduced the accumulation of virus-derived GFP from PVX-GFPΔTGB (Fig. 1c, 1d). We also noticed that At and Nb AGO2 accumulated at similar levels when co-expressed with PVXΔTGB (Fig. 1d) suggesting that P25 may affect the two different AGO2 proteins differently. Consistent with this, expression of AGO2 proteins with P25 alone reduced NbAGO2 accumulation, but not AtAGO2 (Fig. 1e). Taken together, these results suggest that P25 VSR activity is at least partially responsible for the differential efficiency in targeting PVX observed between these two AGO2 proteins.

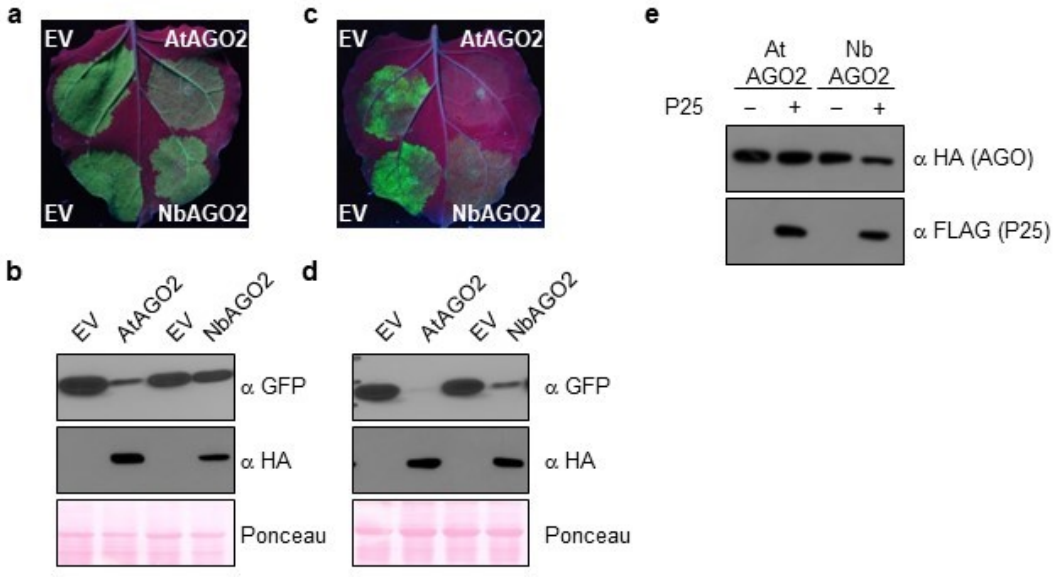

Fig. 1 - AtAGO2, but not NbAGO2, shows anti-viral activity against PVX. a and **c**, *N. benthamiana* leaves were agroinfiltrated with PVX-GFP WT **a** or ΔTGB **c** along with 35S:HA-

AtAGO2, 35S:HA-NbAGO2 or empty vector (EV). Leaves were photographed under UV illumination at 4 days post infiltration (dpi). **b** and **d**, Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in **a** and **c** at 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). HA-tagged AGO proteins were immunoprecipitated from the same extracts and subjected to anti-HA immunoblotting (middle panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. **e**, HA-tagged AGO proteins were co-expressed by agroinfiltration in *N. benthamiana* leaves with either FLAG-tagged P25 or with empty vector (EV). Total proteins were extracted and subjected to anti-FLAG immunoblotting (bottom panel). HA-tagged AGO proteins were immunoprecipitated and subjected to anti-HA immunoblotting (top panel).

4.3.2 The *Arabidopsis AGO2* gene displays a high degree of polymorphism

The NbAGO2 and AtAGO2 proteins are only 50% identical(Nakasugi et al., 2013), which may explain they are being differentially affected by PVX P25 and makes it difficult to identify specific residues important for anti-PVX defense. To evaluate whether the *AGO2* gene shows differences within a species, we analyzed the coding sequences of *AGO1* (At1g48410) and *AGO2* (At1g31280) from 80 *Arabidopsis* accessions, representing eight Eurasian geographic regions(Cao et al., 2011) as obtained from the 1001 genomes project (The 1001 Genomes Consortium, 2016) and by sequencing for some accessions (Supplementary Table 1). Upon analysis, we noticed that multiple alleles possessed short indels (compared to the reference Col-0 allele), mainly located in the 5' end of the *AGO2* coding sequence resulting in extensive variation in the resulting protein lengths, ranging from 993 to 1014 amino acids. We also found a high level of single-nucleotide polymorphisms (SNP) throughout the *AGO2* coding sequence (Fig. 2a and Supplementary Table 1). Synonymous SNPs are six times more frequent in the *AGO2* coding region than that of *AGO1*, while non-synonymous SNPs are more than fifty times more frequent (Fig. 2a), suggesting that *AGO2* has been subjected to selection pressure.

Selective pressures on AGO2 sequences were evaluated by analysing the ratio of nonsynonymous (*dN*) to synonymous substitution rates per site (d*S*). Site-by-site analysis

indicated that residue 33 (Col-0 allele) showed the strongest signal of having undergone positive selection pressure (Fig. 2b). In the 80 accessions analyzed, only two different amino acids are found at position 33, namely an aspartic acid or a glycine (Supplementary Table 1). The Col-0 accession encodes an aspartic acid at position 33 whereas many other accessions, including the commonly used C24 accession, encodes a glycine at the equivalent position (Fig. 2c). Interestingly, the presence of a glycine at residue 33 is almost always correlated with deletions, of variable length ranging from 2 to 13 amino acids, in the region of the protein N-terminal to residue 33, which encodes a number of GR repeats (Figure 2b), as is seen in the C24 accession (Fig. 2c). Given this strong correlation, we refer to *AGO2* alleles encoding 33D as Col-0-like and those encoding 33G plus a GR deletion as C24-like, although C24 *AGO2* possesses 2 additional SNPs, A110D and T131S, and an insertion of a valine at residue 134.

Although multiple AGO2 alleles showed additional indels and SNPs, none of these individual differences were present at high prevalence. We thus classified *AGO2* variants into four groups based on the presence/absence of GR deletions and residue 33, including Col-0-like and C24-like alleles as well as alleles that possessed a complete (Col-0 like) GR motif, but G33 or those with an GR deletion, but D33. In the set of 80 Eurasian *Arabidopsis* accessions Col-0-like and C24-like alleles are present at a frequency of 50% and 43,75%, respectively (Fig. 2d) and both alleles are found in all eight sub-populations (Supplementary Fig. 1a). Only three variants with a full GR motif plus G33 and only two variants with a GR deletion plus D33 were identified, which we refer to collectively as rare *AGO2* alleles (Fig. 2d and Supplementary Fig. 1a). Interestingly, the presence of an aspartic acid at the equivalent of residue 33 of AGO2 appears to be the exception in the *Brassicaceae* family, with other species investigated encoding a glycine at the equivalent residue (Supplementary Fig. 1b).

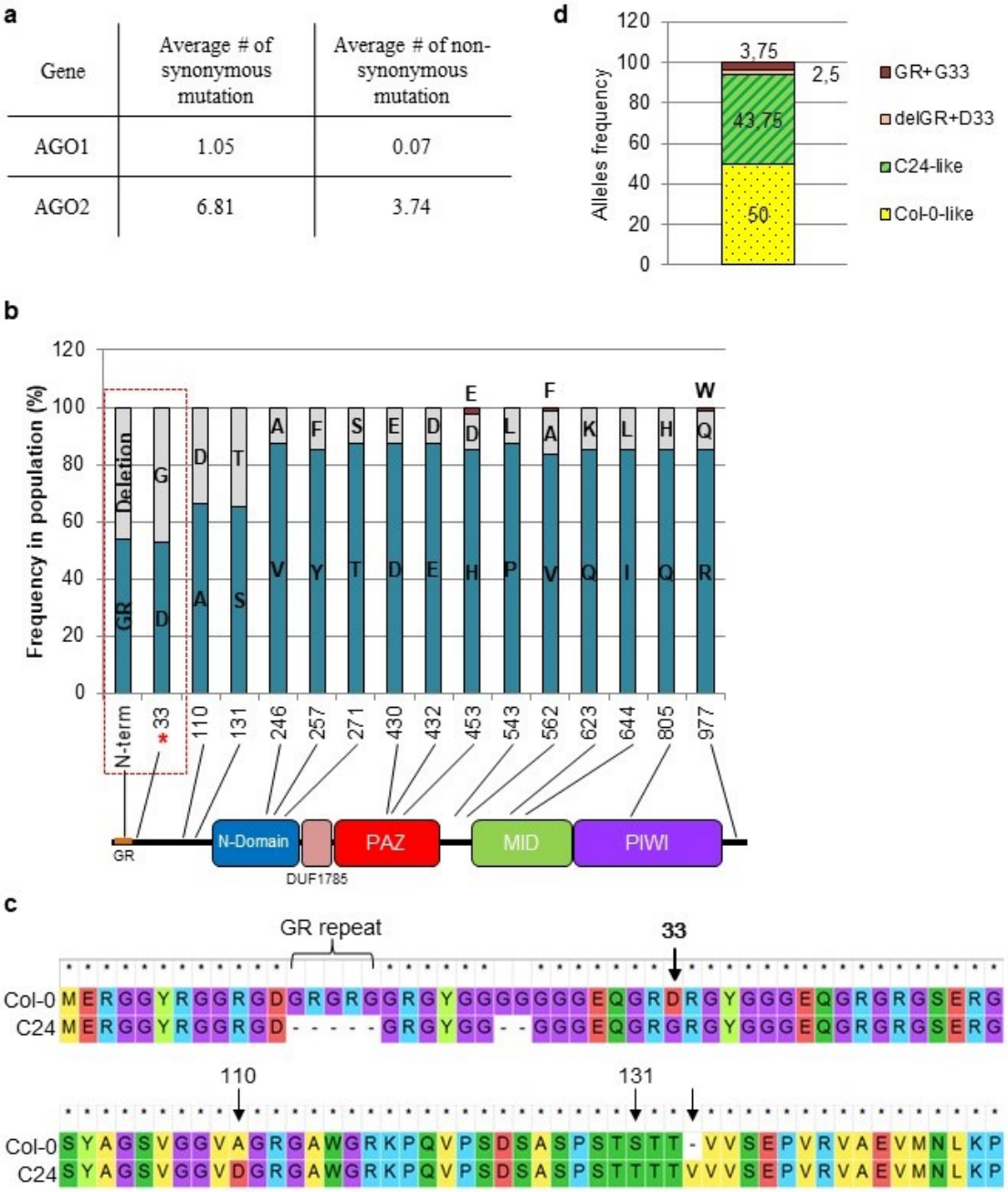

Fig. 2 - Residue 33 of *Arabidopsis AGO2* gene has undergone positive selection in natural populations. a, Variability observed in *AGO1* and *AGO2* sequences in 80 Eurasian *Arabidopsis* accessions in comparison to the Col-0 accession. **b**, Chart showing AGO2 residues having a high frequency of non-synonymous polymorphisms in the 80 accessions. A red dotted rectangle

identifies polymorphisms frequently co-occurring in AGO2. An asterisk indicates an amino acid variant found to be subjected to positive selection pressure with a posterior probability of >95%, supported by fixed-effects likelihood (FEL). Different domains of AGO2 are schematically represented by rectangles (not to scale) under the chart. Blue: N-terminal, red: PAZ, green: MID and purple: PIWI. **c**, Alignment of the N-terminal region of AGO2 protein of Col-0 and C24 AGO2 alleles. **d**, Graph representing allele frequencies in 80 different Eurasian accessions based on polymorphisms found in GR motif and residue 33 of AGO2 N-terminal region.

4.3.3 C24-like AGO2 allele is strongly associated with PVX accumulation in systemic leaves of *Arabidopsis*.

To test whether sequence variation observed in AGO2 might influence antiviral activity, we inoculated 63 accessions from the eight different populations with PVX. Susceptibility or resistance was scored based on the detection of PVX CP in systemic tissues by immune blotting. First, we noticed that resistance or susceptibility of an accession did not correlate with its geographic origin (Fig. 3a; Supplementary Table 1). However, we found that 27 out of 30 tested accessions possessing a Col-0-like *AGO2* were, resistant to PVX (Fig. 3; Supplementary Table 1). Conversely, 24 out of 31 tested accessions having either a C24-like or rare *AGO2* allele were susceptible to PVX (Fig. 3; Supplementary Table 1). The *JAX1* gene has been shown previously to confer broad-spectrum resistance to potexviruses and its presence varies between *Arabidopsis* accessions (Yamaji et al., 2012). We thus determined the *JAX1* status (+/-) in all accessions tested (Fig. 3; Supplementary Table 1). After doing so, we found that all resistant accessions with a C24-like *AGO2* allele, except Copac-1, also possessed a functional *JAX1* gene (Fig. 3). Together, these results indicate that variability in *AGO2* sequence is strongly associated with susceptibility to PVX in *Arabidopsis*.

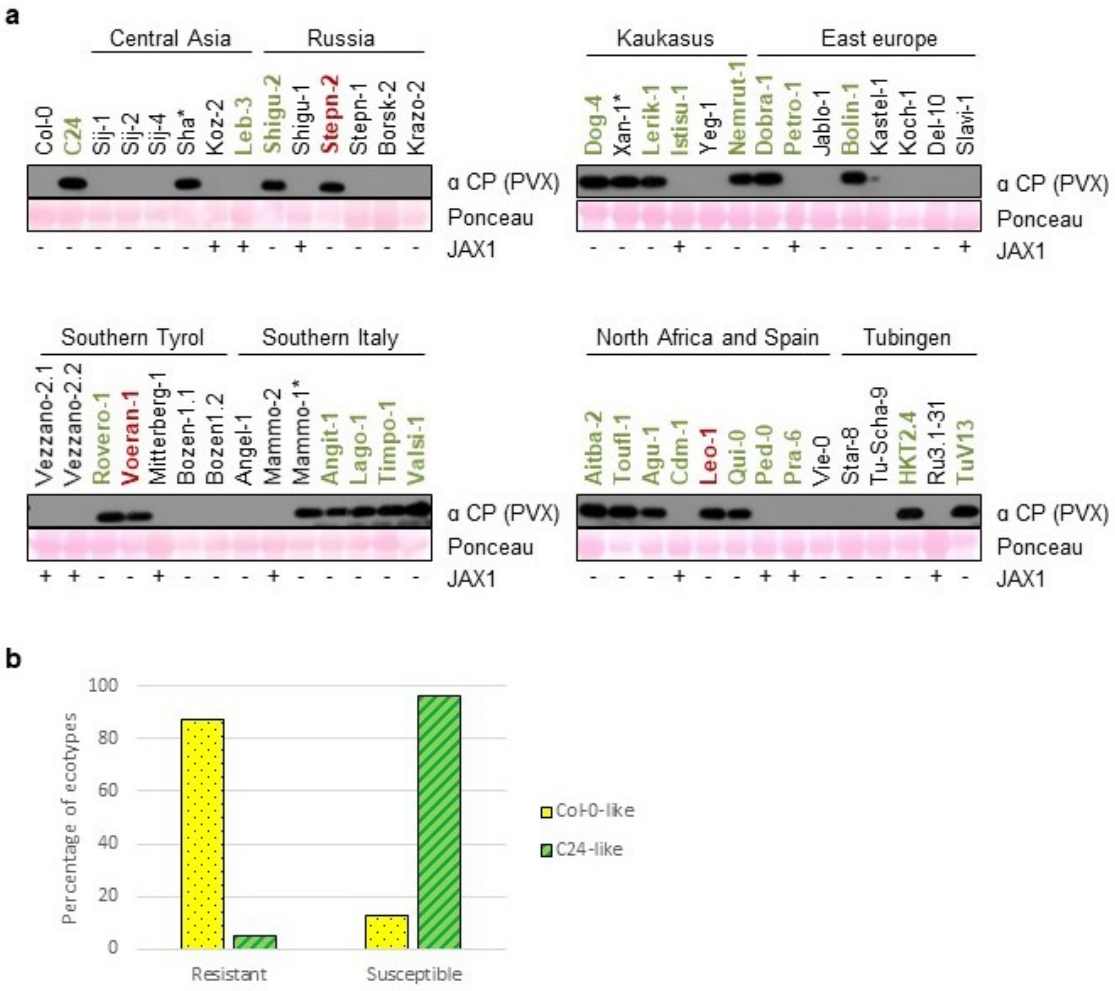

Fig. 3 - Natural variation in the N-terminus of AGO2 correlates with susceptibility to PVX. **a**, Different *Arabidopsis* accessions were inoculated with PVX, as indicated, categorized by geographic region of origin. At 21 dpi, total protein extracts were prepared from systemic leaves and subjected to SDS-PAGE followed by anti-PVX CP immune blotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Accession names are colored according to their AGO2 allele: Col-0-like are black, C24-like are bold green and rare alleles are bold red. Asterisks indicate ecotypes that do not fit in the correlation. Accessions were also genotyped *in silico* for the presence or absence (+/-) of a functional *JAX1* allele. Note that less proteins were loaded into wells for C24, Stepn-2 and Bolin-1 due to the strong accumulation of PVX in these accessions. **b**, Compilation of results obtained for all JAX (-)

ecotypes tested as in **a**. The Pearson's r-coefficient is 0.9958207 and falls within a 95% confidence interval.

4.3.4 Validation of the effect of different AGO2 alleles and susceptibility to PVX in reciprocal inbred lines

Although we observed a strong association between AGO2 and PVX susceptibility, we cannot rule out that polymorphisms in other genes might contribute to the observed phenotypes due to the high level of genetic diversity between the accessions tested(Cao et al., 2011). For this reason, we took advantage of previously described reciprocal introgression lines (RILs). These lines were derived by crossing Col-0 and C24, followed by iterative backcrossing to the parental genotypes and selfing, resulting in lines with a majority of one parental genotype, with relatively small genomic regions derived from the other(Törjék et al., 2008). From this collection, we selected RILs wherein the *AGO2* alleles were exchanged between accessions, as well as control lines wherein genomic regions adjacent to, but not including *AGO2*, were exchanged (Figure 4a and Supplementary Table 2). RILs used in this study are depicted in Figure 4a and the origin of the *AGO2* allele was verified for each line by PCR (Supplementary Fig. 2a). We then assessed these lines for susceptibility to PVX. In agreement with our previous report(Jaubert et al., 2011), no PVX was detected in the systemic tissues of Col-0 but was detected in more than half of the *ago2-1* mutant plants tested (Fig. 4b and Supplementary Fig. 2b). Similarly, PVX was not detected in systemic tissues Col-0 background control RILs N35, N62 and N66. However, PVX was detected in systemic leaves of more than half of the plants from N37 and N55 lines wherein the C24 *AGO2* allele has been introgressed into a Col-0 background (Fig. 4b). Conversely, introgression of the Col-0-like *AGO2* allele into the C24 background abolished susceptibility to PVX in the majority of plants tested (Fig. 4b and Supplementary Fig. 2b). A Pearson's r-coefficient test has shown a statistically significant positive correlation between the AGO2 identity of the plant and its accumulation of PVX in systemic leaves (Fig. 4c).

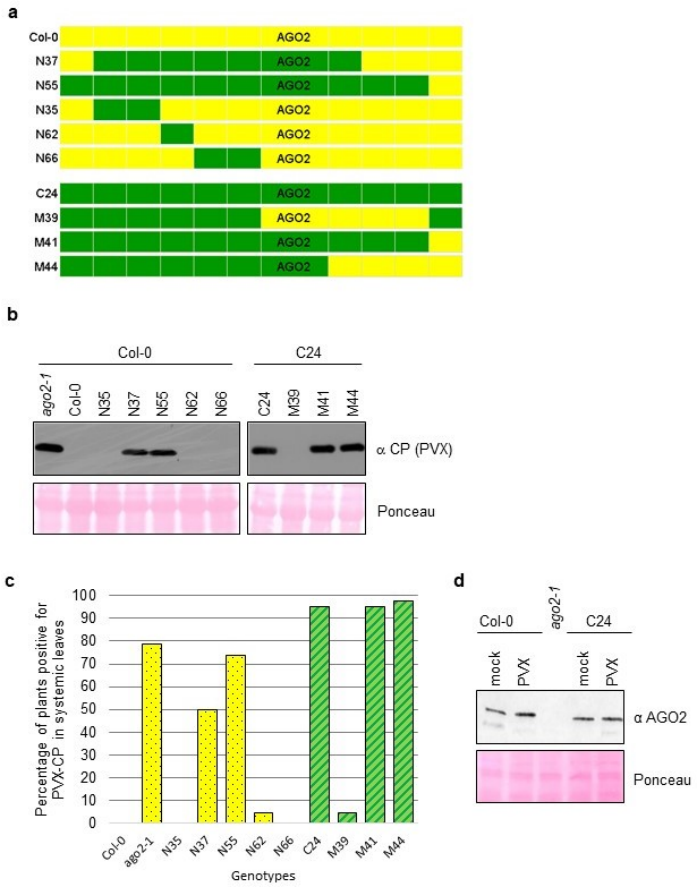

Fig. 4 - Exchange of AGO2 allele between Col-0 and C24 changes susceptibility to PVX. a, Schematic representation of chromosome I in recombinant intogression lines (RILs) from a cross between Col-0 and C24 (Törjék et *al*., 2008). N lines have a Col-0 background (yellow) throughout the genome except for small regions of chromosome I substituted with the corresponding region from C24 (green). M lines have a C24 background (green) with Col-0 substitutions (yellow). **b**, RILs, as depicted in **a**, were inoculated with PVX. At 21 dpi, total proteins extract from systemic leaves were prepared and subjected to SDS-PAGE followed by anti-PVX CP immune blotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. Representative results of the 42 replicates is shown. **c**, Compilation of results obtained for all replicates tested as in **b** (related to Supplementary Fig. 2b). The Pearson's r-coefficient is 0.6140612 and falls within a 95% confidence interval. **d**, Col-0, WT or *ago2-1*, and C24 plants were inoculated with PVX. At 21 dpi, total protein extracts

from systemic leaves were prepared and subjected to SDS-PAGE followed by anti-AGO2 (Agrisera antibody) immune blotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

Exogenous application of the phytohormone salicylic acid has been shown to compromise PVX accumulation in *N. benthamiana*(Naylor et al., 1998) and *ICS1,* a key salicylic acid (SA) biosynthesis gene, is significantly up-regulated in C24 compared to Col-0(Yang et al., 2015). However, quantification of SA in different RILs showed that introgression of the Col-0 genomic region containing the *AGO2* gene into the C24 background, or the opposite exchange, does not significantly change SA accumulation in these lines, relative to the parental genotypes (Supplementary Fig. 3c) thus precluding a role for SA in the observed phenotypes. Moreover, the differential susceptibility cannot be attributed to differential AGO2 expression as both accessions display similar AGO2 protein accumulation in systemic leaves upon inoculation with PVX (Fig. 4d). These results further validate the involvement of AGO2 as a major determinant of resistance of to PVX in *Arabidopsis*.

4.3.5 C24 *AGO2* is not a null allele

In *Arabidopsis*, AGO2 has also been implicated in antibacterial defense responses, presumably through its binding to endogenous miRNAs, as well as in the methylation of some DNA loci(Pontier et al., 2012; Zhang et al., 2011). Aside from the differences found in the N-terminus outlined above, the C24 AGO2 protein does not differ from Col-0 in any of the well-characterized functional AGO domains. To determine whether C24 AGO2 is still efficient in non- virus-related functions, we inoculated RILs with virulent *Pseudomonas syringae* pv. tomato (Pto). In Col-0 background lines in which C24 *AGO2* has been introgressed, namely N37 and N55, Pto grew at titres similar to WT and at significantly lower titers compared to *ago2-1* (Col-0 background) mutant plants (Fig. 5a). Likewise, introgression of Col-0 *AGO2* into C24 had no effect on bacterial growth compared to WT C24 (Fig. 5a). This suggests that C24 *AGO2* polymorphisms do not compromise the function of this protein in response to bacterial

infection. Furthermore, we observed that Pst infection induces *AGO2* expression at similar level in both accessions (Fig. 5b), consistent with previous reports(Zhang et al., 2011).

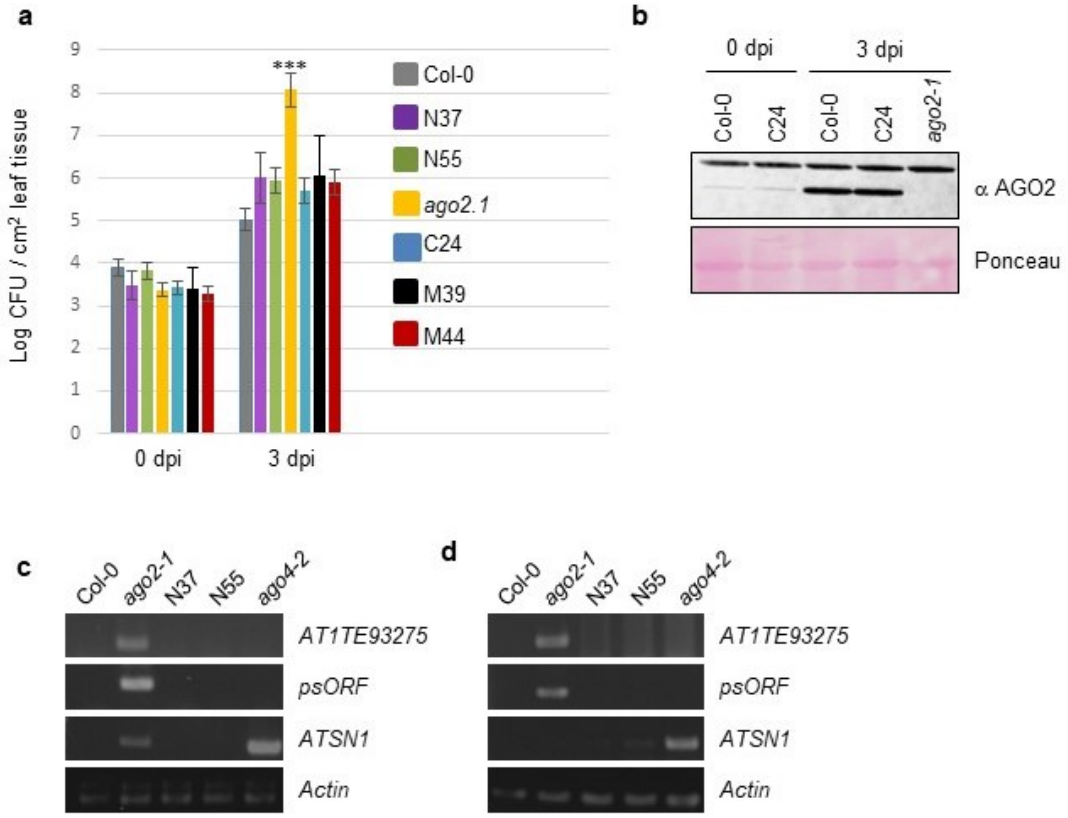

Fig. 5 - The C24-like AGO2 allele retains anti-bacterial and methylation-related functions. **a**, The indicated RILs with Col-0 and C24 backgrounds were infected with virulent Pst. Bacteria were counted at 0 and 3 dpi. Error bars indicate SEM from three biological replicates. Asterisks indicates statistically significant differences (student t test) at a P-value < 0.005. **b**, Total protein extracts were prepared from *Arabidopsis* leaves inoculated as in (A) at 0 and 3 dpi and subjected to SDS-PAGE, followed by anti-AGO2 (Abiocode) immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. **c**, Genomic DNA from the indicated genotypes was isolated and subjected to digestion with McrBC and subjected to PCR with primers to the indicated loci. **d**, RNA from the indicated genotypes was isolated and subjected to RT-PCR using primers to the indicated loci.

A previous study has reported that DNA methylation and gene silencing of the psORF and AT1TE93275 transposable elements are affected in *ago2* mutant plants(Pontier et al., 2012). To examine whether C24 AGO2 is still functional in this respect, DNA methylation status and expression of AT1TE93275 and psORF were verified in two Col-0 lines containing the C24 *AGO2* allele. Genomic DNA from Col-0, *ago2-1*, N37, N55 and *ago4-2* plants was isolated and digested with the methylation dependent restriction enzyme McrBC. As shown in Figure 5c, the *ago2-1* mutant shows amplification of psORF and AT1TE93275 after McrBC digestion, whereas the N37 and N55 lines do not, while all lines show similar amplification of the actin gene (Fig. 5c). As expected, a similar test showed greater amplification of ATSN1, a signature locus for AGO4-dependent methylation, in the *ago4* mutant (Fig. 5c). At the same time, RT-PCR analysis showed that psORF and AT1TE93275 could be amplified only from RNA extracted from the *ago2-1* mutant and ATSN1 showed significant amplification only in the *ago4-2* mutant (Fig. 5d). These results are highly consistent with previous reports(Pontier et al., 2012) and indicate that both the Col-0 and the C24 AGO2 proteins are functional for AGO2-dependent methylation and its associated repression of a transposable element. Taken together, these results suggest that a complete GR motif and D33 of the Col-0 AGO2 protein are required for optimal antiviral defense but appear to be dispensable for regulating endogenous transcripts and methylation-related functions.

4.3.6 C24-AGO2 display decrease antiviral activity against PVX WT compared to that of Col-0-AGO2.

Manual alignment of NbAGO2 and AtAGO2 proteins shows that NbAGO2 encodes a glycine at the position equivalent to G33 of C24-AGO2 (Supplementary Fig. 3). Because NbAGO2 was found to be efficiently antiviral only against a mutated version of PVX (Fig. 1a, 1b, 1c, 1d), we verified whether it behaved similarly to C24-AGO2 in a transient overexpression assay. Indeed, in transient assays, C24-AGO2 is as efficient as Col-0-AGO2 at restrict PVX-GFPΔTGB but is less efficient against WT PVX-GFP (Supplementary Fig. 4a, 4b, 4c, 4d). This phenotype was also seen with other Col-0-like and C24-like AGO2 alleles, Yeg-1 and Bolin-1 respectively (Supplementary Fig. 4e, 4f, 4g, 4h). Furthermore, expressing the different AGO2 variants

under the native NbAGO2 promoter enhance the differential activity of these proteins against WT PVX (Fig. 6a, 6b) as well as against PlAMV-GFP, another Potexvirus (Supplementary Fig. 5a, 5b).

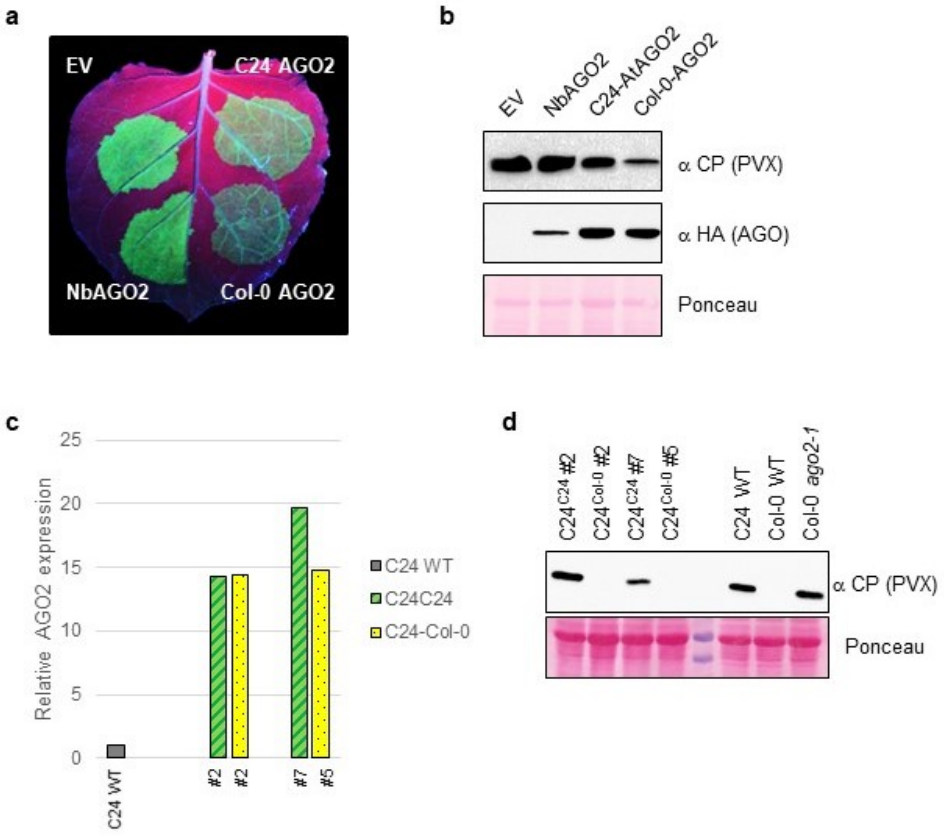

Fig. 6 - Polymorphisms found in C24-*AGO2* affect its antiviral activity in *Arabidopsis*.

a, *N. benthamiana* leaves were agroinfiltrated with PVX-GFP WT along with pNbAGO2:HA-NbAGO2, pNbAGO2:HA-AtCol-0-AGO2, pNbAGO2:HA-AtC24-AGO2 or empty vector (EV). Leaves were photographed under UV illumination at 4 dpi. **b**, Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in **a** at 4 dpi and subjected to SDS-PAGE, followed by anti-PVX CP (top panel) or anti-HA (middle panel) immune blotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading. **c** and **d**, C24 WT or transgenic plants were inoculated with PVX WT. At 21 dpi, systemic leaves were harvested for qRT-PCR analysis **c** and immune blotting **d**. **c**, Total RNA was extracted from systemic leaves and subjected to qRT-PCR analysis to determine relative expression levels

of total AGO2. Relative expression of AGO2 in transgenic lines, $C24^{C24}$ and $C24^{Col-0}$, has been normalized with relative expression to that of C24 WT. **d**, Total protein extracts from systemic leaves were prepared and subjected to SDS-PAGE followed by anti-PVX CP immune blotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

To verify whether this differential antiviral activity between Col-0-AGO2 and C24-AGO2 is biologically relevant, we created C24 transgenic lines expressing Col-0-AGO2 or C24-AGO2 under the control of the 35S promoter. AGO2 transgene expression was then monitored by qRT-PCR as, despite being under the control of a strong promoter, both proteins were undetectable by immune blotting analysis (data not shown). Lines with similar AGO2 expression level upon PVX inoculation ($C24^{C24}$#2 and $C24^{Col-0}$#2) were analyzed by immune blotting to verify their susceptibility to PVX (Fig. 6c, 6d). At 21 dpi, PVX was not detected in systemic leaves of $C24^{Col-0}$ lines showed whereas $C24^{C24}$#2 showed similar accumulation of PVX compared to that of C24 WT. In addition, the $C24^{C24}$#7 line, expressing almost 20X more AGO2 than in C24 WT, remains susceptible to PVX as determined by immunoblotting (Fig. 6c, 6d). These results suggest that polymorphisms found in C24-AGO2 compromised its antiviral activity. It also suggests a fundamental role of AGO2 in resistance/susceptibility phenotype observed in different *Arabidopsis* accessions.

4.4 Discussion

4.4.1 Susceptibility to PVX is common in *Arabidopsis thaliana*

In this study, we screened different *Arabidopsis* accessions for PVX susceptibility and showed that, although the widely used *Arabidopsis thaliana* model accession, Col-0, is resistant to PVX and can restrict PVX accumulation to inoculated leaves, a number of wild type accessions are susceptible (Fig. 3). This correlates with recent studies showing, first of all, that this plant species possesses Potexvirus-specific factors to support PVX replication(Hashimoto et al., 2016; Jaubert et al., 2011; Keima et al., 2017) and secondly, that different non-host resistance

factors contribute to the PVX-resistance phenotype observed in Col-0(Jaubert et al., 2011) or PlAMV-resistance phenotype observed in some other accessions(Yamaji et al., 2012).
AGO2 has been shown previously to be an important player in antiviral RNA silencing and to confer resistance to PVX in *Arabidopsis* Col-0(Jaubert et al., 2011), so natural variation in the coding sequence of this protein was analyzed in this paper. This has established a strong correlation between the presence of a C24-AGO2 allele (deletion in RG and/or 33G) and the susceptibility to PVX.

4.4.2 High prevalence of polymorphisms in AGO2 coding sequence

Different surveys of wild *A. thaliana* accessions revealed extensive natural allelic variation comprising SNPs as well as indels(Koornneef et al., 2004; Nordborg et al., 2005; Zhang et al., 2008). These naturally occurring variations has often been associated with resistance to various biotic factors(Koornneef et al., 2004; Todesco et al., 2010). Here, we have shown that the antiviral AGO2 protein is well reflecting this variation. Effectively, AGO2 coding region has 50X more non-synonymous SNPs than the AGO1 coding region (Fig. 2a). Similarly, a study in Drosophila has shown that genes related to antiviral RNAi evolve significantly at a faster rate than paralogous gene implicated in housekeeping RNAi functions (miRNA pathway)(Obbard et al., 2006). Because of its essential role in antiviral defense response against different RNA viruses(Carbonell and Carrington, 2015), it is not surprising to see such variation in AGO2. Indeed, since every virus encodes for at least one VSR, some of which requires interaction with RNAi proteins, host antiviral RNAi components must rapidly adapt to win the molecular arms race against pathogen(Mukherjee et al., 2013; Obbard et al., 2006). Moreover, a study demonstrated that viruses have driven close to 30% of all adaptive amino acid changes, making them dominant drivers of protein adaptation in mammals(Enard et al., 2016). Importantly, in this study, no polymorphism was found in residues already identified to be important for sRNA loading and maturation, RNA binding, AGO hook or catalytic functions(Fátyol et al., 2016). Although variation has been observed throughout the coding sequence of AGO2, the only residue found to be under positive selection was in the N-terminus, that is to say, outside of conserved functional domain (Fig. 2) similarly to what was observed in mammal RNAi

genes(Obbard et al., 2006). Although Nordborg et al. suggested that such tests for selection may not be appropriate for *Arabidopsis* because of its recent population growth(Nordborg et al., 2005), our data for *Arabidopsis* AGO2 are similar to what was observed in animal systems. Finally, this study underpins the importance of non-conserved domains or regions without described function. Because they are not directly implicated in function of the protein, these domains may be allowed to evolve more easily to counteract pathogens attack.

4.4.3 Variation in *AGO2* N-terminus portion is an important virus resistance determinant

Although PVX does not infect *Arabidopsis thaliana* Col-0 WT, these plants possess the cellular machinery compatible with Potexvirus replication, including PVX(Hashimoto et al., 2016; Jaubert et al., 2011; Keima et al., 2017). By challenging different *Arabidopsis thaliana* accessions, we observed a strong correlation between the allele an accession has and its ability to restrict the accumulation of the virus (Fig.3) although we cannot rule out that additional factors may be implicated. In line with this, we found three accessions, with a Col-0-like AGO2, that were susceptible to PVX (Fig. 3a). Moreover, we observed that three accessions with a C24-like AGO2 (C24, Bolin-1 and Stepn-2) were more susceptible to PVX than others, to such an extent that the amount of protein loaded for immune blotting had to be reduced by one-third (Fig. 3a). Taken together, these results strongly suggest that other genes are most likely implicated in the outcome of PVX-Arabidopsis interaction and that, some of them, could have a dominant negative effect over a Col-0-like AGO2 allele.

Because of the high natural variation between accession, the numbers of accessions analysed here did not allow to identify AGO2 in a GWAS analysis (data not shown). However, the PVX-inoculated RILs lines between Col-0 and C24 accessions, a resistant and a susceptible accession respectively, narrow down the PVX- susceptibility phenotype to a region in chromosome I (Fig.4). Several genes in this region display polymorphisms including AGO3. Phylogenetically, AGO3 is the closest homologue to AGO2 however, except against BaMV, AGO3 has not been found to be required for virus resistance(Alazem et al., 2017; Brosseau and Moffett, 2015; Brosseau et al., 2016; Garcia-Ruiz et al., 2015; Jaubert et al., 2011), but rather appears to play a role in DNA methylation(Zhang et al., 2016). Moreover, although C24 AGO3 possesses some

polymorphisms, *in silico* analysis revealed that these polymorphisms are also found in resistant accessions (Supplementary Table 2) precluding its implication in PVX susceptibility phenotype. Transient overexpression assays in *N. benthamiana* revealed that C24-AGO2 and C24-like-AGO2 possess antiviral activity although to a lesser extent than Col-0-AGO2 and Col-0-like AGO2 (Supplementary Fig. 4). However, when stably overexpressed in *A. thaliana* C24, C24-AGO2 does not confer resistance to PVX whereas Col-0-AGO2 does (Fig. 6c, 6d). We hypothesize that the antiviral activity of C24-AGO2 may be sufficiently affected to allow PVX to escape this defense response and infect systemically *Arabidopsis*. Alternatively, it is possible that a co-factor of AGO2, present in *N. benthamiana* but not in Arabidopsis, could contribute to the anti-PVX functionality of C24-AGO2 in *N. benthamiana*. Although further experiments are required to characterize how C24-AGO2 is affected, our data reinforce the idea that polymorphisms found in the N-terminus of AGO2 strongly modulate antiviral activity efficiency in *Arabidopsis* genetic background (Fig. 6c, 6d).

4.4.4 Possible functional significance of AGO2 polymorphisms

One of the most frequent polymorphism in the AGO2 of susceptible accessions is found in the N-terminus, at position 33 (Fig. 2b, Fig. 3). Most of susceptible accessions analyzed possess G33 whereas the resistant ones possess D33. Aspartic acid is a negatively charged amino acid. Further experiments are needed to determine if this negative charge is sufficient to modulate the antiviral activity of AGO2, for example, by altering the protein-protein interactions.

The N-terminus of AGO2 encode a number of GR repeats previously identified as Block 43, a block that is conserved between animal and plant AGO(Rodríguez-Leal et al., 2016). Although well conserved, this block is present in different copy numbers in different AGO proteins in different species. Among *Arabidopsis* AGO proteins, AGO1, 2, 3, 5 and 10 encode multiple B43 motifs(Rodríguez-Leal et al., 2016). Among these, only AGO2 and AGO5 can target PVX WT, although only AGO1 is destabilized by the PVX-P25 suggesting that the presence of this block is not sufficient to confer PVX antiviral activity(Brosseau and Moffett, 2015). In numerous animal systems, arginine residue within this block has been shown to be methylated

by PMRT(Kirino et al., 2009, 2010; Musiyenko et al., 2012; Siomi et al., 2010; Vagin et al., 2009). Because of the conservation of the block between animals and plants, it is tempting to speculate that plant AGOs possessing this block43 undergo the same post-translational modification (PTM) and that deletions, present only in susceptible accessions, may impair this PTM. This post-translational modification has been shown to modulate protein-protein interaction and protein localization and stability(Kirino et al., 2009, 2010; Musiyenko et al., 2012; Siomi et al., 2010; Vagin et al., 2009). It will be interesting to determine the role of these GR repeat in antiviral activity of AGO2 and whether they undergo such modifications. However, as both Col-0 and C24 seem to accumulate at similar level (Fig. 4, Fig. 5, Fig. 6), and because both AGO2 have similar antibacterial and DNA methylation activities (Fig. 4), we rule out the possibility that these polymorphisms do not impair protein stability or sRNAs loading.

4.4.5 Two recurrent polymorphisms to counteract the two PVX-P25 functions

In this study, we identified what we called 'rare alleles' which possess or deletion in GR repeats or G33. Interestingly, accessions possessing these alleles are also susceptible to PVX (Fig. 3) suggesting that these two polymorphisms independently affect antiviral activity against PVX. The PVX-P25 has been shown to have dual anti-silencing activity by first, destabilizing AGO proteins and second, by forming tight VRCs(Brosseau and Moffett, 2015; Chiu et al., 2010; Tilsner et al.). Both important polymorphisms identified in this study, namely the GR repeats and residue 33, are found in the N-terminus of AGO2. To date, no function as been attributed to this portion of AGO proteins. However, it has been speculated that N-terminus forming a coil structure in some Arabidopsis AGO may be important for association with membrane and that is interacting with PIWI domain(Poulsen et al., 2013). Although AGO2 does not encodes for such coil domain, it is also tempting to speculate that its N-terminus, even if different from AGO1 and AGO7, may serve for similar function. First, because only the antiviral acitivity, and not antibacterial and methylation function, is compromised in C24-AGO2, we predicted that these polymorphisms would not affected interaction between N-terminus and PIWI domain as such modification would dramatically change the protein structure and thus, the overall function. In the other hand, as PVX has been shown to form very tight ER-membrane-bound

VRC that are thought to protected viral RNAs from AGO antiviral functions, it would be very interesting to test whether polymorphisms found *Arabidopsis thaliana* AGO2 proteins modulate their capacity to enter into the VRC(Brosseau and Moffett, 2015; Tilsner et al.). Because resistant accessions possess an aspartic residue, a negatively charged amino acid, at position 33, it is possible that this charge may prevents P25 VSR activity on Col-0-AGO2. Alternatively, this negative charge could contribute to the recruitment of additional factors required for antiviral defense.

4.5 Conclusion

Half of the accessions analyzed possess an ineffective AGO2 allele for PVX defence in *Arabidopsis thaliana*. For an allele to be conserved along evolution, it needs to confer some advantage(Mauricio, 1998; Todesco et al., 2010). Various possibilities are being considered. First, it is possible that C24-AGO2 allele is functional against naturally occurring virus in *Arabidopsis* populations. Alternatively, it is possible that a Col-0-like AGO2 has a negative effect on *Arabidopsis* fitness. Such conservation of a non-fonctional allele in antiviral silencing has been described with *RDR1* gene in *N. benthamiana*(Bally et al., 2015). In this specific case, it was established that a functional antiviral allele negatively impacts early vigour in *N. benthamiana*(Bally et al., 2015).

A recent study showed that AGO2 has undergone positive selection in tomato during the domestication process(Koenig et al., 2013). A number of economically important tomato cultivars are highly susceptible to Potexvirus, particularly to Pepino mosaic virus (PepMV)(Candresse et al., 2010). In regard to this, it would be interesting to investigate whether introgression of a Col-0-like AGO2 allele in tomato would confer resistance to PepMV.

4.6 Methods

4.6.1 Plant material and growth conditions

Nicotiana benthamiana and *Arabidopsis thaliana* plants were grown on soil (BM6, Berger and Agromix, Fafard PLACE, respectively) in growth chambers with 16-h-light/8-h-dark and 12-h-light/8-h-dark photoperiod at 23°C and 21°C respectively. Col-0 (CS28168), C24 (CS28127) and *Arabidopsis* wild accessions (CS76427) come from ABRC stock center. The *ago2-1* mutant in Col-0 background has been described elsewhere(Takeda et al., 2008). RILs between Col-0 and C24 were kindly provided by R.C. Meyer and have been described previously(Törjék et al., 2008).

4.6.2 Plasmid construction and transient expression

The *NbAGO2* ORF was cloned using cDNA derived from TBSV infected *N. benthamiana* leaves using primers listed in Supplemental Table 3. For the generation of different *Arabidopsis* AGO2 expression clones, cDNA (Figure 1) and gDNAs (Figure 6, Supplemental Figure 4 and Supplemental Figure 5) of the appropriate accessions were used as templates for PCR amplification with primers listed in Supplemental Table 3. PCR products were purified and cloned into the pGEM-T easy vector (Promega) and subcloned into the XbaI and BamHI sites of pBIN61 vector containing an N-terminal FLAG epitope in frame with the XbaI site or in pBIN61 empty vector for HA-tagged NbAGO2. All other constructions have been previously described including PVX, PVX-GFP, PVX-GFPΔTGB and PlAMV-GFP binary constructs(Bhattacharjee et al., 2009; Lu et al., 2003; Peart et al., 2002; Yamaji et al., 2012), FLAG-P25(Brosseau and Moffett, 2015), pBIC-HA-AtAGO2(Takeda et al., 2008). For the generation of AGO2 expression vector under the control of the NbAGO2 promotor, AGO2 upstream regulatory sequences (2068 pb) were amplified from genomic DNA with primers listed in Supplemental Table 2. PCR products were purified and cloned into the pGEM-T easy vector (Promega) and subcloned into the Acc65I and XbaI sites and BamHI sites of pBIN61

vector to replace the 35S promoter. Agrobacterium-mediated transient expression assays in *N. benthamiana* were performed as previously described(Brosseau and Moffett, 2015).

4.6.3 Virus inoculation

Infections of three-week-old *Arabidopsis thaliana* plant were carried out by rubinoculation as previously described(Brosseau and Moffett, 2015). Briefly, saps were produced with PVX-infected *N. benthamiana* plant material. Infected material was ground in 0.1 M phosphate buffer, pH 7.0 (2 mL/g of infected tissue). Mock inoculations were performed with sap produced with uninfected *N. benthamiana* plants (2 mL/g of healthy tissues).

4.6.4 Protein extraction and analysis

Protein extraction and analysis were carried out as previously described(Brosseau and Moffett, 2015). Proteins were detected by immune blotting using anti-HA-horseradish peroxidase conjugated (HRP) antibodies (Sigma, 1:3,000 dilution), anti-FLAG-HRP antibodies (Sigma, 1:5,000 dilution), anti-GFP-HRP antibodies (Santa Cruz, 1:3,000 dilution), anti-PVX-CP rabbit polyclonal antibodies (Agdia, 1:3,000 dilution) and anti-AGO2 antibody (Agrisera, 1:5,000 dilution or Abiocode 1:7,500). Detection of the latter three primary antibodies was performed using donkey anti-IgG rabbit-HRP polyclonal antibodies (BioLegend, 1:10,000 dilution).

4.6.5 Gene expression and DNA methylation analysis

Total RNA was isolated with Trizol (Ambion) and subjected to RT-PCR using primers listed in Supplemental Table 2. Gene expression and McrBC analyses were performed as previously described(Pontier et al., 2012) with minor modifications. 10 µg of genomic DNA were digested

with 6u of McrBC (NEB) in a final volume of 50 µl for 3h at 37°C. The digested DNA was then analyzed by semi-quantitative PCR using primers listed in Supplementary Table 3.
RT-qPCR were done as previously described(Brosseau et al., 2016; El Oirdi et al., 2011) using primers listed in Supplementary table 3.

4.6.6 Quantification of SA

Quantification of SA was performed by PhenoSwitch Bioscience Inc. (Sherbrooke, Canada). Briefly, salicylic acid was extracted from crushed tissues by the addition of 500µl of methanol containing 0.01 ng/µl of internal standard (D4-Salicylic acid). The samples were then incubated at 4°celcius for 30 minutes with end-over-end mixing and the insoluble material was cleared by centrifugation. The supernatant was diluted 10 fold in water and the pH was adjusted to 7 by the addition of 50 mM ammonium acetate. A standard curve containing 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.91, 1.95 or 0.97 µg/ml SA was prepared in 50 µl H2O and processed the same way as the plant samples. For the weak anion exchange solid phase extraction (WAX SPE) of the salicylic acid and its internal standard, the protocol was the following: phase conditionning with acetonitrile, wash with 50 mM ammonium acetate, sample loading, wash with 50 mM ammonium acetate, elution with 5% ammonium hydroxyde in water. The eluate was dried down by speed vac, reconstituted in 50µl of water containing 0.2% formic acid and 10 mM ammonium formate and processed by LC-MS/MS. As some samples were too concentrated, they were diluted 10-fold and reanalysed by LC-MS.

4.7 References

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4.9 Contributions

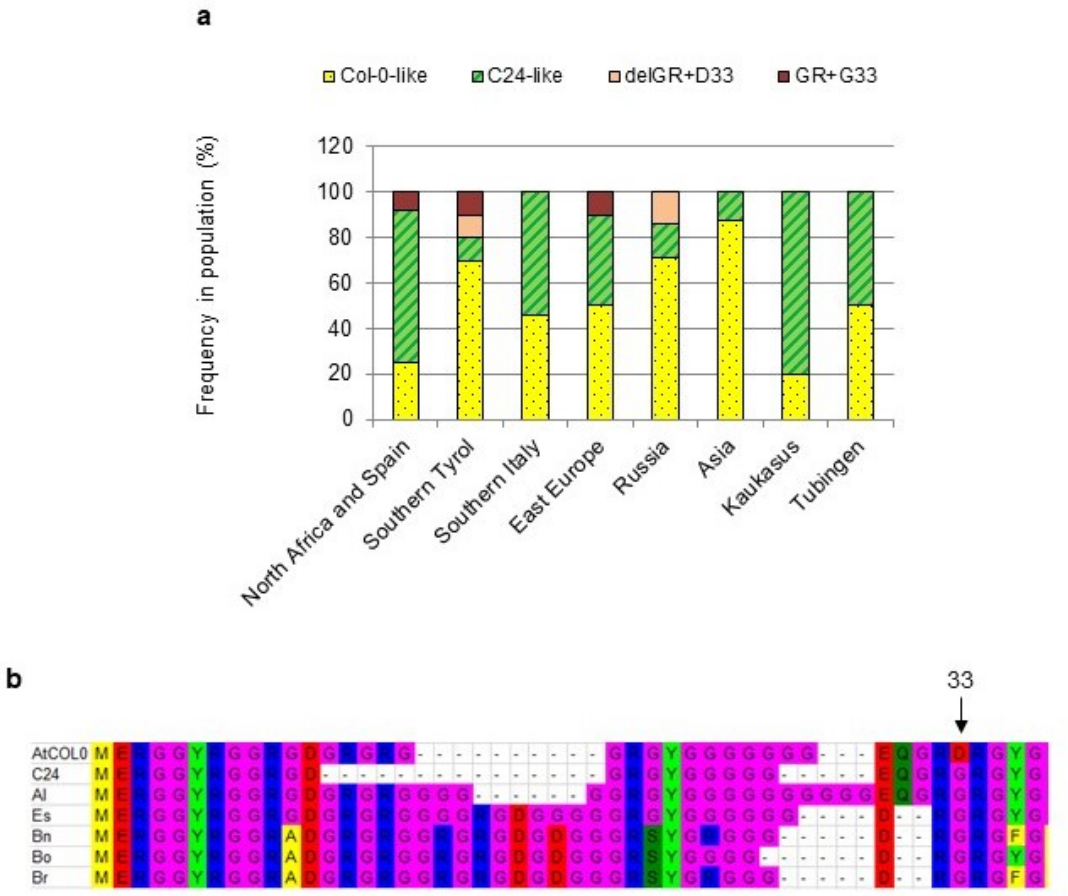
C.B. and P.M. conceived and designed the experiments. C.B., A.A., C.R.L., Z.Z. and S.B. performed the experiments. C.B. and A.A. analyzed the data. C.B. and P.M. wrote the article.

4.10 Competing interests

The authors declare no competing interests.

4.11 Supplementary information

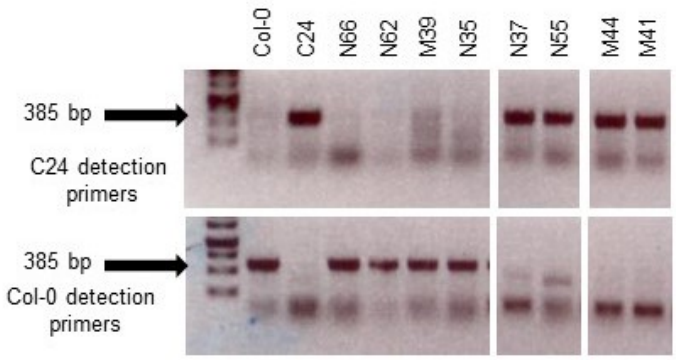
4.11.1 Supplementary figures



Supplementary Fig. 1 - C24 and Col-0 alleles are found in all eight Eurasian populations analyzed.

a, Chart representing allele frequencies in eight different Eurasian populations. Note that alleles have been classified based only on polymorphisms found in the GR motif and residue 33 of AGO2 regardless of polymorphisms found elsewhere in the coding sequence. **b**, Amino acid alignment of a portion of the N-terminus of AGO2 from *Arabidopsis thaliana* Col-0 (AtCol-0), C24 (At-C24) and *Arabidopsis lyrata* (Al) as well as others *Brassicaceae*, *Eutrema salsugineum* (Es), *Brassica napus* (Bn), *Brassica oleracea* (Bo) and *Brassica rapa* (Br).

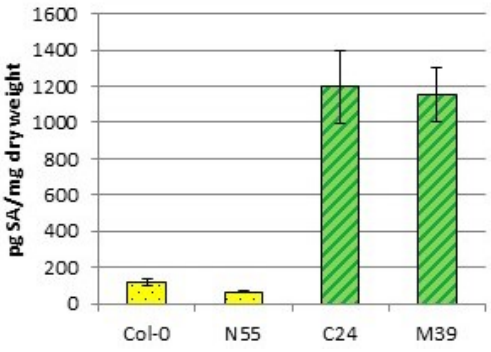
a



b

Background	Line	AGO2 allele	PVX in systemic leaves
Col-0	WT	Col-0	0/42
	ago2-1	none	33/42
	N35	Col-0	0/42
	N37	C24	21/42
	N55	C24	31/42
	N62	Col-0	2/42
	N66	Col-0	0/42
C24	WT	C24	40/42
	M39	Col-0	2/42
	M41	C24	40/42
	M44	C24	41/42

c

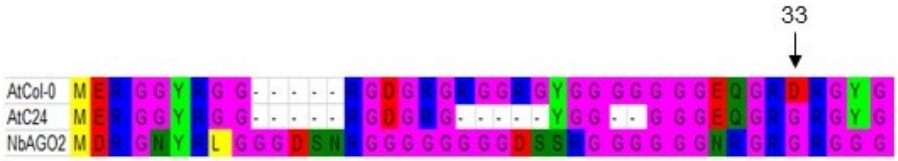


Supplementary Fig. 2 - Correlation between *AGO2* allele and susceptibility of *Arabidopsis* to PVX.

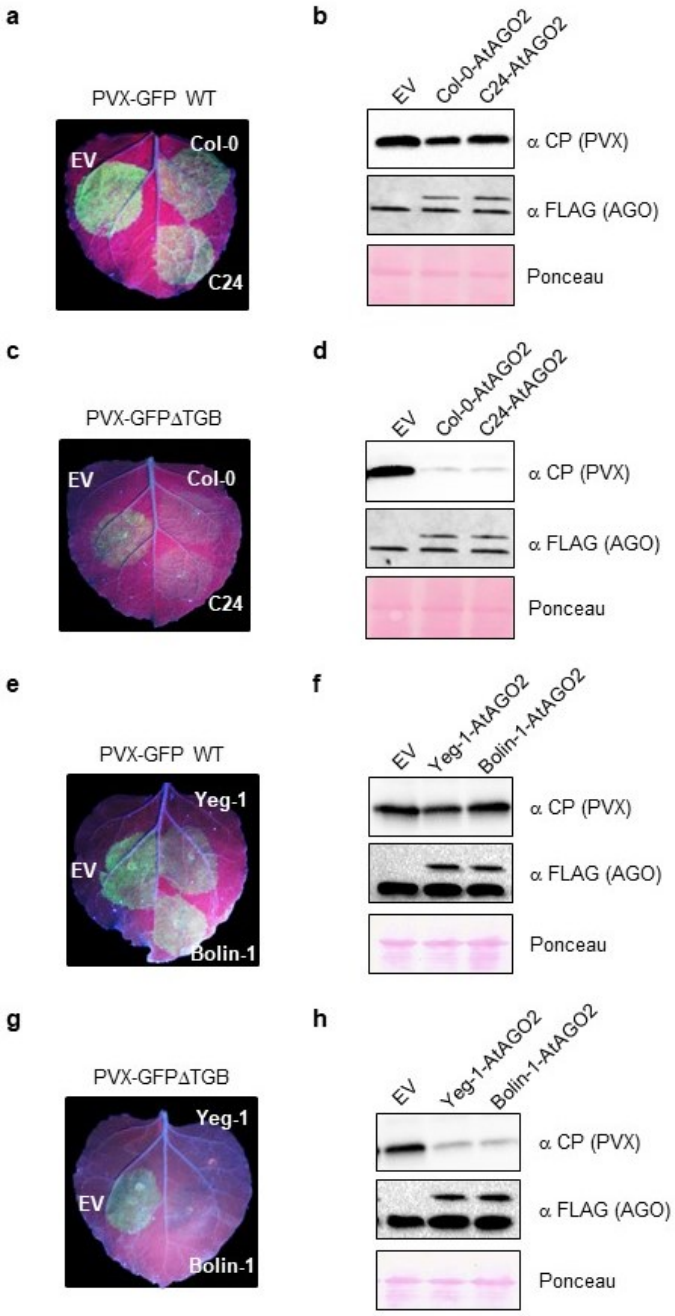
a, Validation of AGO2 alleles by PCR reaction on gDNA of RILs with allele-specific primers.

b, Number of PVX-inoculated plants showing systemic infection were scored by anti-PVX CP

immunoblotting at 21 dpi. **c**, Quantification of SA content by LC-MS/MS in two different RILs and their respective background accessions, namely Col-0 and C24.



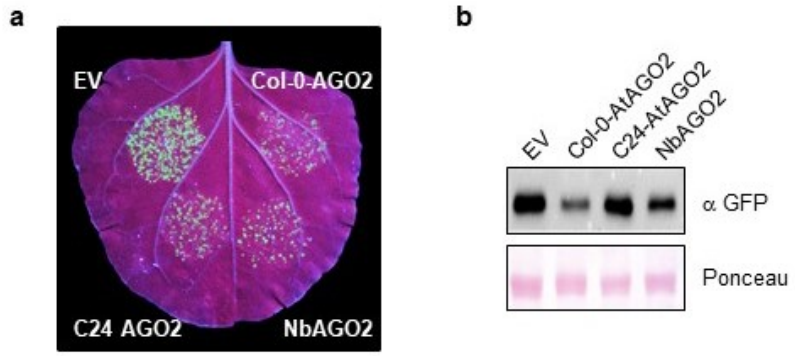
Supplementary Fig. 3 - Amino acid alignment of a portion of the N-terminus of AGO2 from *Nicotiana benthamiana* (NbAGO2) and *Arabidopsis thaliana* Col-0 (AtCol-0) and C24 (AtC24).



Supplementary Fig. 4 - Polymorphisms found in C24- and C24-like- *AGO2* affect its antiviral activity.

a, c, e, g, *N. benthamiana* leaves were agroinfiltrated with PVX-GFP WT **a** and **c** or ΔTGB **e** and **g** along with 35S:FLAG-AtCol-0-AGO2, 35S:FLAG-AtC24-AGO2 or empty vector (EV) **a** and **c** or with 35S:FLAG-AtYeg-1-AGO2, 35S:FLAG-AtBolin-1-AGO2 or empty vector

(EV) **e** and **g**. Leaves were photographed under UV illumination at 4 dpi. **b**, **d**, **f, h**, Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in **a**, **c**, **e**, **g** at 4 dpi and subjected to SDS-PAGE, followed by anti-PVX CP (top panel) or anti-FLAG (middle panel) immune blotting. Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.



Supplementary Fig. 5 - The difference in antiviral activity observed between the different alleles is also observed against PlAMV.

a, *N. benthamiana* leaves were agroinfiltrated with PlAMV-GFP along with 35S:FLAG-AtCol-0-AGO2, 35S:FLAG-AtC24-AGO2, 35S:HA-NbAGO2 or empty vector (EV). Leaves were photographed under UV illumination at 4 days post infiltration (dpi). **b**, Total protein extracts were prepared from *N. benthamiana* leaves agroinfiltrated as in **a** at 4 dpi and subjected to SDS-PAGE, followed by anti-GFP immunoblotting (top panel). Ponceau staining (bottom panel) of the same extracts is shown to demonstrate equal loading.

Supplemental Table 1. Description of ecotypes. Polymorphisms found in AGO2 sequence of different ecotypes (in comparison to Col-0 AGO2. Accessions marked with Δ are those whose first exon of AGO2 has been sequenced. *GR represents a polymorphism in copy number of GR repeat and/or repeat unit sequence. Presence of full length JAX1 is indicated as Yes whereas the presence of a premature stop codon is indicated as No. --- indicates that public available sequences of this region does not allowed to conclude about presence/absence of a premature stop codon in JAX1 sequence. Number of plants showing systemic infection were scored by anti-PVX CP immunoblotting at 21 dpi (last column). nt indicated an accession that has not been tested.

Origin	Seed stock #	Abbreviated name	Non-Synonymous SNP	JAX1 (At1G58160)	Presence of PVX in systemic leaves
Central Asia	CS76379	Sij-1	NONE	No	0/12
	CS76380	Sij-2	NONE	No	0/12
	CS76381	Sij-4	NONE	No	0/12
	CS76382	Sha Δ	NONE	No	4/12
	CS76383	Koz-2	R43L/V562F	Yes	0/10
	CS76384	Kly-4	NONE	No	Nt
	CS76385	Kly-1	NONE	Yes	Nt
	CS76426	Leb-3	*GR/D33G/R977Q	Yes	0/10
Russia	CS76374	Shigu-2	*GR/D33G/R34C/A110D/S131T	No	4/12
	CS76375	Shigu-1	NONE	Yes	0/10
	CS76376	Kidr-1	NONE	---	Nt
	CS76377	Stepn-2 Δ	*GR	No	11/16
	CS76378	Stepn-1 Δ	NONE	No	0/16
	CS76421	Borsk-2	NONE	No	0/10
	CS76422	Krazo-2	NONE	No	0/7

Supplemental Table 1. (suite)

Origin	Seed stock #	Abbreviated name	Non-Synonymous SNP	JAX1 (At1G58160)	Presence of PVX in systemic leaves
Southern Tyrol	CS76349	Vezzano-2.1	R977Q	Yes	0/16
	CS76350	Vezzano-2.2	R977Q	Yes	0/16
	CS76351	Rovero-1 Δ	*GR/D33G/A110D/S131T/K312R	No	9/12
	CS76352	Voeran-1	D33G/R977Q	No	7/12
	CS76353	Altenb-2	*GR/S131T	No	Nt
	CS76354	Miterberg-1 Δ	R977Q	Yes	0/16
	CS76355	Castelfed-4-212	R977Q	Yes	Nt
	CS76356	Castelfed-4-213	R977Q	Yes	Nt
	CS76357	Bozen-1.1	NONE	No	0/9
	CS76358	Bozen-1.2	NONE	No	0/9
Southern Italy	CS76359	Ciste-1	G18D/A110D	No	Nt
	CS76360	Ciste-2	S131T/P138A/R977Q	No	Nt
	CS76361	Monte-1	G18D/A110D/S131T	No	Nt
	CS76362	Angel-1	G27E/S980L	No	0/12
	CS76363	Moran-1	*GR/D33G/A110T/S131T	Yes	Nt
	CS76364	Mammo-2	*GR/D33G/A110D/S131T/E432D/L504V	Yes	0/14
	CS76365	Mammo-1Δ	G18D/A110D/S131T	No	1/14
	CS76366	Angit-1	*GR/D33G/A110D/S131T/E432D	No	5/12
	CS76367	Lago-1	*GR/D33G/A110D/S131T/R774C	No	7/12
	CS76368	Apost-1	*GR/D33G/V246A/Y257F/S271T/D430E/H453D/P543L/V562A/Q623K/I644L/Q805H	No	Nt
	CS76423	Galdo-1	NONE	No	0/6
	CS76424	Timpo-1	*GR/D33G/A110D/S131T	No	7/12
	CS76425	Valsi-1	*GR/D33G/A110D/S131T/ E432D	No	7/14

Supplemental Table 1. (suite)

Origin	Seed stock #	Abbreviated name	Non-Synonymous SNP	JAX1 (At1G58160)	Presence of PVX in systemic leaves
Kaukasus	CS76386	Dog-4 Δ	*GR/D33G/A110D/S131T/V221M/E432D/G433R/ T702I	No	10/16
	CS76387	Xan-1 Δ	A110D/S131T/R709L/R774C	No	3/9
	CS76388	Lerik1-3	*GR/D33G/R977Q	No	9/12
	CS76389	Istisu-1	*GR/D33G/A110D/S131T/E432D/G433R/T702I/M955V	Yes	0/12
	CS76390	Lag2-2 Δ	D33G/R977Q	No	2/9
	CS76391	Vash-1	*GR/D33G/R977Q	No	2/9
	CS76392	Bak-2	*GR/D33G/A110D/S131T/E432D	No	Nt
	CS76393	Bak-7	*GR/D33G/V246A/Y257F/S271T/D430E/H453D/P543L/V562A/K589Q/Q623K/I644L/Q805H	No	Nt
	CS76394	Yeg-1 Δ	P93L	No	0/16
	CS76398	Nemrut-1	*GR/D33G/A110D/S131T/V221M/E432D/G433R/ T702I	No	11/16
East Europe	CS76369	Dobra-1	*GR/D33G/V246A/Y257F/S271T/D430E/H453D/ P543L/V562A/Q623K/I644L/Q805H	No	6/9
	CS76370	Petro-1	*GR/D33G/V246A/Y257F/S271T/D430E/H453DP543L/V562A/Q623K/I644L/Q805H	Yes	0/9
	CS76371	Lecho-1	D33G/D738N	No	Nt
	CS76372	Jablo-1	G18D/S980L	No	0/9
	CS76373	Bolin-1 Δ	*GR/D33G/A110D/S131T	No	15/16
	CS76395	Kastel-1	G52E	No	0/9
	CS76396	Koch-1	L681M	No	0/9
	CS76397	Del-10 Δ	V246A/Y257F/S271T/D430E/P543L/V562A/Q623A/644L/ Q805H	No	0/16
	CS76419	Slavi-1	NONE	Yes	0/12
	CS76420	Copac-1	*GR/D33G/R70W/V246A/Y257F/S271T/D430E/H453D/P543L/V562A/Q623KI644L/Q805H	No	0/6

Supplemental Table 1. (suite)

Origin	Seed stock #	Abbreviated name	Non-Synonymous SNP	JAX1 (At1G58160)	Presence of PVX in systemic leaves
North Africa and Spain	CS76347	Aitba-2	*GR/D33G/A110D/A114V/A126P/S131T	No	8/12
	CS76348	Toufl-1	*GR/D33G/A110D/A114V/A126P/S131T	No	7/12
	CS76409	Agu-1	*GR/D33G/V246A/Y257F/S271T/D430E/H453D/ P543L/V562A/Q623K/I644L/Q805H/	No	5/9
	CS76410	Cdm-0	*GR/D33G/A110D/S131T/E432D	Yes	0/9
	CS76411	Don-0	*GR/D33G/V246A/Y257F/S271T/D430E/H453D P543L/V562A/Q623K/I644L/Q805H	Yes	Nt
	CS76412	Fei-0	G18D/A92T/A110D/S131T	No	Nt
	CS76413	Leo-1 Δ	D33G/A110D/V121F/S131T/Y183F/V209I/M233P/ Y257F/R266Q/H453D/R559K/V562A/A592V/Q623K/I644 L/Q805H	No	7/12
	CS76414	Mer-6	R977W	No	0/3
	CS76415	Ped-0	*GR/D33G/A110D/V121F/S131T/Y183F/V209I/M23P/Y2 57F/H453D/V562A/R559K/A592V/Q623K/I644L/Q805H	Yes	0/9
	CS76416	Pra-6	*GR/D33G/R34C/A110D/Q120H/S131T	Yes	0/9
	CS76417	Qui-0	*GR/D33G	No	4/6
	CS76418	Vie-0	S360L/N847S	No	0/6
Tubingen	CS76399	Ey1.5-2	*GR/D33G/V246A/Y257F/S271T/D430E/H453D/P543L/V 562A/P569L/Q623K/I644L/Q805H	No	Nt
	CS76400	Star-8	NONE	No	0/12
	CS76401	Tu-Scha-9	NONE	No	0/12
	CS76402	Nie1-2	P138A/R977Q	No	0/6
	CS76403	Tu-SB30-3	*GR/D33G/A110D/S131T/E432D/E435K/H453E/	No	Nt
	CS76404	HKT2-4	*GR/D33G/V246A/Y257F/S271T/D430E/P543L/V562A/Q 623K/I644L/Q805H	No	6/12
	CS76405	Tu-Wa1-2	*GR/D33G/R34C/A110D/Q120H/S131T	No	4/12
	CS76406	Ru3.1-31	L681M	Yes	0/12
	CS76407	Tu-V-13	*GR/D33G/A110D/S131T/R774C	No	9/12
	CS76408	Wal-HasB-4	P89T/R977Q	No	Nt

Supplementary Table 2a. ***In silico*** **verification of the presence of a number of genes involved in RNA silencing pathway and / or in Potexvirus-specific recessive resistance in the genomic region exchanged in the RILs tested.** M39 (C24 background, heterozygous for markers between $MASC05303_{chrI}$ and $MASC03340_{chrI}$); N37 (Col-0 background, heterozygous for markers between $MASC09203_{chrI}$ and $MASC02577_{chrI}$); N55 (Col-0 background, heterozygous for markers between $MASC03771_{chrI}$ and $MASC04209_{chrI}$).

Gene name	TAIR identifier	Present in the intervalle in M39, N37 **AND** N55	Polymorphism at amino acid level in C24	Correlation genotype/phenotype
AGO1	At1G48410	No	No	n/a
AGO2	**At1G31280**	**Yes**	**Yes**	**Yes** (Fig 3b)
AGO3	**At1G31290**	**Yes**	**Yes**	**No**
AGO4	At2G27040	No	n/a	n/a
AGO5	At2G27880	No	n/a	n/a
AGO6	At2G32940	No	n/a	n/a
AGO7	At1G69440	No	n/a	n/a
AGO8	At5G21030	No	n/a	n/a
AGO9	At5G21150	No	n/a	n/a
AGO10	At5G43810	No	n/a	n/a
DCL1	At1G01040	No	n/a	n/a
DCL2	At1G03300	No	n/a	n/a
DCL3	At3G43920	No	n/a	n/a
DCL4	At5G20320	No	n/a	n/a
RDR1	At1G14790	No	n/a	n/a
RDR2	At4G11130	No	n/a	n/a
RDR6	At3G49500	No	n/a	n/a
DRB1	At1G09700	No	n/a	n/a
DRB2	At2G28380	No	n/a	n/a
DRB3	At3G26932	No	n/a	n/a
DRB4	At5G20320	No	n/a	n/a
DRB5	At5G41070	No	n/a	n/a
SGS3	At5G23570	No	n/a	n/a
EXA1	At5G42950	No	n/a	n/a
CBP (eIF4E)	At5g18110	No	n/a	n/a

Supplementary table 2b. Analysis of *AGO3* coding sequence shows no correlation between polymorphisms and susceptibility/resistance phenotype observed in different ecotypes. In each of the 8 regions, 2 ecotypes, without JAX1, were randomly selected (1 susceptible and 1 resistant) giving priority to ecotypes that have been tested several times. *GR represents a polymorphism in copy number of GR repeat and/or repeat unit sequence.

Susceptible ecotypes	Polymorphisms found in AGO3
C24	L246P/A830T/F1190V
SHA	L246P/K335M/F1190V
Stepn2	P137S/Q161R/G162D/R217K/L246P/V842L/ F1190V
Rovero-1	L246P/V842L/T1066M/S1171F/F1190V
Lago-1	L246P/S540P/F1190V
Nemrut-1	L246P/F1190V
Bolin-1	L246P/A315S/K381R/ Q567K/K763N/ F1190V
Aitba-2	*GR/V108A/G124D/P137Q/Q161R/G162D/R169K/ D178G/V194G/R217K/R233Q/V236I/L246P/G272A Q567K/ F1190V
Tu-V-13	L246P/A985P/F1190V
Resistant ecotypes	Polymorphisms found in AGO3
Col-0	None
Sij-1	L246P/F1190V
Stepn-1	P156S/D226G/L246P/F1190V
Bozen1.1	G114S/ L246P/E904G/F1190V
Angel-1	L246P/F1190V
Yeg-1	None
Del-10	L246P/A315S/K381R/Q567K/K763N/F1190V
Vie-0	L246P/T855S/F1190V
Star-8	L246P /L532F/Q567K/ F1190V

Supplementary Table 3. Primers list used in this study.

Primer Name	Sequence	Use
AtAGO2-Fwd	TCTAGAGCCACCATGGAGAGAGGTGGTTATCG	Transient assay
HA-AtAGO2-Fwd	TCTAGAGCCACCATGTACCCATACGATGTTCCTGACTATGCGATGGAGAGAGGTGGTTATCG	Transient assay
AtAGO2-Rev	GGATCCTCAGACGAAGAACATAACATTCTC	Transient assay
HA-NbAGO2-Fwd	CCTAGGATGGACTACAAAGACGATGATGACAAGTCTAGAATGGATCGTGGAAATTACC	Transient assay
HA-NbAGO2-Rev	GGATCCTCAGACAAAGAACATTATGTTC	Transient assay
Col-0-AGO2-Fwd	GTTCAGTAGGAGGAGTCGC	Validation of AGO2 allele in RILs
C24-AGO2-fwd	CGGTTCAGTAGGAGGTGTCGA	Validation of AGO2 allele in RILs
AGO2-Rev	GCTAAGGGAAACTCATCGGG	Validation of AGO2 allele in RILs
AtSN1-RTPCR-Fwd	ACCAACGTGCTGTTGGCCCAGTGGTAAATC	Gene silencing status (RT-PCR)
AtSN1-RTPCR-Rev	AAAATAAGTGGTGGTTGTACAAGC	Gene silencing status (RT-PCR)
AtSN1-McrBC-Fwd	TGTCTTGGAAAGGATATTGGAAG	DNA methylation status (McrBC)
AtSN1-McrBC-Rev	AAGTGGTGGTTGTACAAGCC	DNA methylation status (McrBC)
AT1TE93275-McrBC-Fwd	ATGAAGGAGAAGAGACGAGT	DNA methylation status (McrBC)
AT1TE93275-McrBC-Rev	TCGGAACCAATCGGTTTCAC	DNA methylation status (McrBC)
At3g18780-McrBC-Fwd	GCCATCCAAGCTGTTCTCTC	Gene silencing status (RT-PCR)/ DNA methylation status (McrBC)
At3g18780-McrBC-Rev	CCCTCGTAGATTGGCACAGT	Gene silencing status (RT-PCR)/ DNA methylation status (McrBC)
NbProm-Fwd	ggtaccCTATTTGCTCTTGTTCTTGAG	Transient assay
NbProm-Rev	tctagaTTTCCAACTTCACTAAAGAG	Transient assay
qPCR-TotalAGO2-Fwd	taggaggagtcgctggtagagg	Transgenics analysis – total AGO2 expression
qPCR-TotalAGO2-Rev	caccgatggcttcagattcata	Transgenics analysis – total AGO2 expression
eIF2alpha-Fwd	gagtctcttcttgaggcacttc	Transgenics analysis - Housekeeping
eIF2alpha-Rev	gctggatcatccttggagttag	Transgenics analysis - Housekeeping

CHAPITRE 5

DISCUSSION ET CONCLUSION GÉNÉRALE

L'étude de l'implication de l'ARN interférence dans la défense antivirale semblait chose simple tel que présenté dans la littérature aux débuts de mes études. Les 10 protéines ARGONAUTE chez *Arabidopsis* sont classées dans trois clades distincts selon leur homologie de séquence. À ce moment, on croyait fortement que cette homologie de séquence conférait des fonctions fortement similaires (Vaucheret et al., 2006). De plus, l'analyse du patron d'expression ainsi que l'analyse de la localisation sub-cellulaire de certaines de ces protéines AGOs pouvaient nous laisser croire que l'on pouvait éliminer l'implication de certaines de ces protéines dans la défense antivirale. Par exemple, dans la défense antivirale, on pouvait quasiment déjà éliminer AGO5, une protéine dont l'expression n'avait été détectée que dans les tissus reproducteurs et ce, même en condition de stress biotiques ou abiotiques (Schmid et al., 2008; Kapoor et al., 2008; Tucker et al., 2012). D'autres parts, une protéine AGO nucléaire telle qu'AGO4 avait bien peu de chance d'intervenir dans la PTGS dirigée contre un virus à ARN. Néanmoins, les résultats de mes travaux me permettent de croire que l'ARN interférence est bien plus compliqué que s'on l'imaginait au départ! Il semble qu'une seule protéine AGO ait souvent de multiples fonctions chez les plantes et, par conséquent, le placement traditionnel des protéines AGO dans la voie TGS ou PTGS basée sur l'analyse phylogénétique de la similarité des séquences pourrait être inexacte et une nouvelle classification s'impose.

Dans le premier article, nous avons démontré l'implication d'AGO5 dans la défense contre PVX, un Potexvirus. Nous avons aussi démontré que son expression est induite dans les tissus systémique lors d'une infection efficace par PVX i.e., lors d'une infection chez le mutant *dcl2 dcl3 dcl4* et chez le mutant *ago2*. Il y a deux informations importantes ici : (1) son expression est seulement dans les tissus distants de l'infection primaire (2) seulement lorsqu'il y a une forte accumulation de virus dans les feuilles inoculées. Ces résultats suggèrent qu'il y a une induction

d'un signal, fort probablement produit dans les feuilles inoculées, qui se propage dans les feuilles non-inoculées via le système vasculaire pour sonner l'alarme et préparer une défense contre l'envahisseur. Tel que décrit précédemment, les plantes possèdent en effet un moyen de protéger les feuilles distales du lieu primaire d'infection contre l'agent pathogène. C'est la résistance systémique acquise (Kachroo and Robin, 2013; Spoel and Dong, 2012). Mais quel pourrait être le signal dans le cas de l'interaction Arabidosis-PVX-AGO5? S'il s'agit bien de SAR ici, le signal pourrait bien être des hormones végétales tel que SA, JA ou Et. L 'implication dans la SAR de ces hormones, souvent modifiées, est bien définie. D'autres parts, nous avons aussi vu précédemment que la boucle d'amplification de l'ARN interférence permet le mouvement de cellule-en-cellule mais aussi le mouvement dans les tissus systémiques des petits ARNs. Une forte accumulation de petits ARNs pourrait être le 'signal' permettant d'induire l'expression d'AGO5.

Dans ce premier article, nous avons aussi démontré que toutes les protéines AGOs possèdent la capacité intrinsèque de reconnaître et cibler les vARNs de PVXΔTGB. Les essais de complémentation de PVXΔTGB avec la co-expression de la P25 en *trans* semblent indiquer que la P25 permet de protéger les vARNs de deux façons : (1) par son activité VSR permettant de déstabiliser certaines protéines AGOs (AGO1 et AGO7) (2) par son rôle central dans la formation du VRC de PVX qui permettrait de protéger les vARNs en excluant la machinerie de l'hôte. D'ailleurs, dans le cas de la P25 de PVX, cette seconde activité semble être plus importante que l'activité VSR puisqu'elle permet de compromettre l'activité antivirale de plusieurs protéines AGOs non-déstabilisées par la P25 (AGO3, AGO4, AGO6, AGO9, AGO10). Des études précédentes ont démontré que l'on peut découpler ces deux activités en faisant des mutations ponctuelles dans la P25 de PVX (Baynes et al., 2005; Yan et al., 2012; Tilsner et al., 2012). Pour valider l'hypothèse que la formation du VRC est fondamentale pour protéger l'ARN de PVX , il serait intéressant de vérifier l'activité antivirale des AGOs contre des variants de PVX dont la P25 a été mutée pour sa fonction d'organisateur des VRC mais qui est toujours fonctionnelle dans son activité VSR (VRC(-) et VSR(+)); et inversement un PVX ayant une P25 VSR(-) et VRC(+).

Dans le second article, nous avons démontré qu'AGO4 était l'AGO la plus efficace pour ralentir PlAMV, un autre Potexvirus. Nous avons montré qu'AGO4 est relocalisée dans le cytoplasme lors d'une infection par PlAMV. Dans les essais transitoires, nous avons montrés que le mutant AGO4ΔNLS possède encore son activité antivirale contre PlAMV suggérant que la relocalisation était bénéfique pour la cellule. Mais quel signal peu bien induire cette relocalisation? Notre première hypothèse est l'abondance de vsiARNs (et de leurs cibles) dans le cytoplasme. En effet, on sait qu'une AGO4 non-associée à un sARN se trouve dans le noyau. Lors du chargement du petit ARN, il y a probablement un changement de conformation qui démasque le NLS et induit ainsi la localisation au noyau. Deux hypothèses sont plausibles ici : (1) AGO4 ayant chargée un vsiARNs est 'séquestrée' dans le cytoplasme par l'appariement entre le petit ARN et sa cible. (2) La prédominance des sARNs de 21- et 22-nt lors d'une réponse antivirale pourrait empêcher AGO4 de s'associer avec ses sARNs habituels, les hc-siARNs de 24-nt.

Un point important conclu de ses deux études est que malgré que deux virus fassent partie du même genre, ici les Potexvirus, et qu'ils encodent donc chacun un VSR, ici la P25, ayant fort probablement des fonctions similaires; ces deux virus ne seront pas ciblés par les mêmes protéines antivirales. Notre hypothèse ici est au niveau du complexe de réplication virale (VRC). Effectivement, PVX qui est ciblé par très peu de protéines AGO (2 et 5) malgré qu'il possède un VSR faible, laisse croire qu'il conçoit un VRC plus structuré qui empêche les AGOs d'accéder à son génome. À l'inverse, malgré que PlAMV encode un VSR fort capable de déstabiliser plusieurs protéines AGO, il est ciblé par plus de protéines AGO comparativement à PVX. Ce qui laisse croire que son génome est facilement accessible (Figure 1).

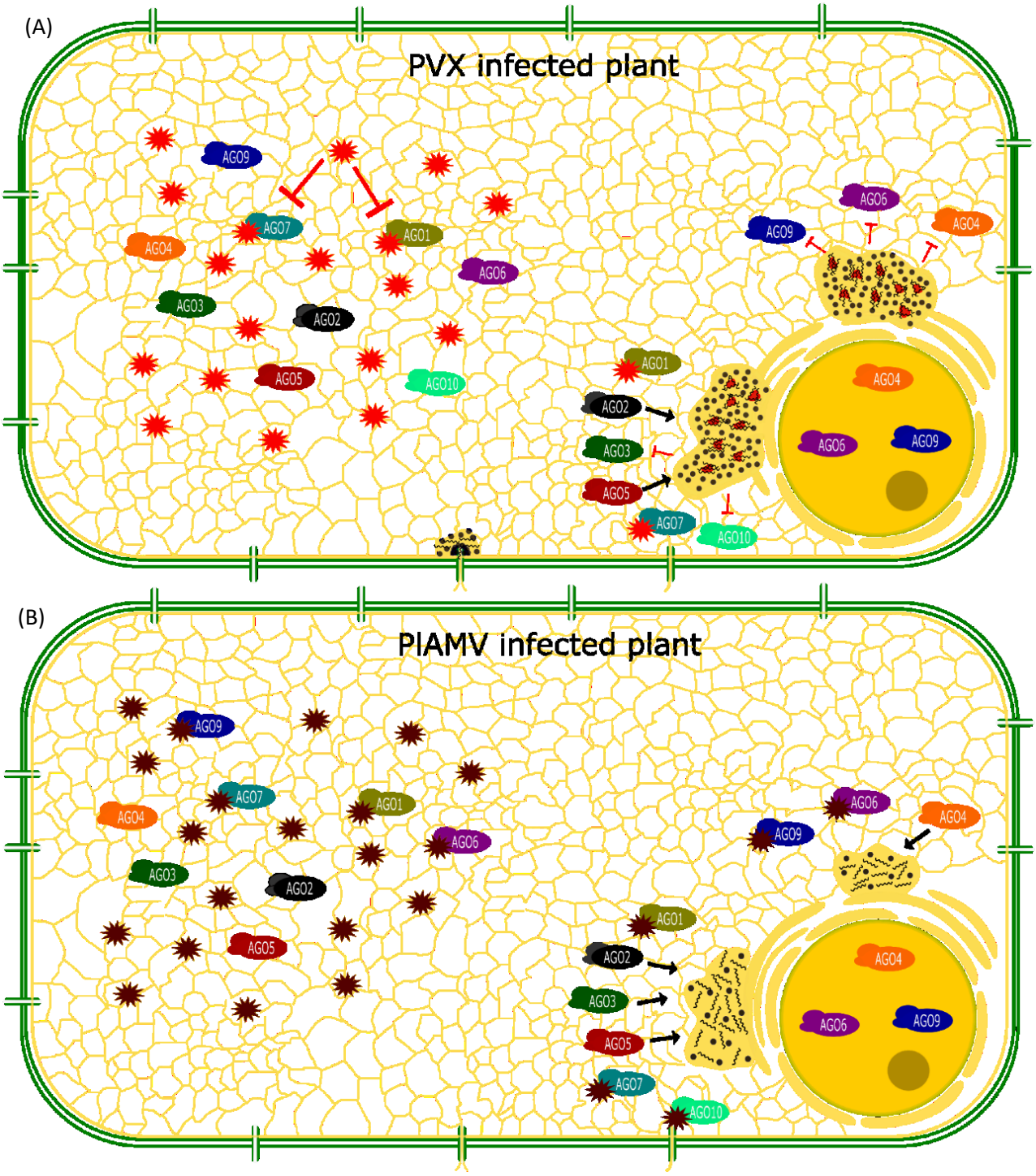


Figure 1. Modèle proposé pour les AGOs antivirales contre les Potexvirus (Figure tirée de Silva-Matins, Adurgogbangba and Moffett en rédaction) (A) Les neuf protéines AGO d'*Arabidopsis* testées pour leur activité antivirale contre PVXΔTGB ou PVXΔP25 sont capable

de ciblées l'ARN de PVX induisant ainsi une diminution de l'accumulation des protéines virales. Néanmoins, lorsque ces mêmes essais ont été répétées avec un PVX sauvage, possédant la P25, seules AGO2 et AGO5 permettent de restreindre l'accumulation du virus suggérant que la P25 est un facteur important pour protéger le virus contre les mécanismes de défense de l'hôte. L'activité de la P25 de PVX peut être décortiquée en deux volets : activité VSR permettant de compromettre la stabilité d'AGO1 et d'AGO7; et son rôle crucial pour la formation des X-bodies périnucléaire associés à la réplication permettant de protéger les ARN viraux d'être ciblés par AGO3, AGO4, AGO6, AGO9 et AGO10. (B) Parmi les neuf protéines AGO d'Arabidopsis testées pour leur activité antivirale contre PlAMV, toutes les protéines qui ne sont pas déstabilisées par la P25 de PlAMV (AGO2, AGO3, AGO4 et AGO5) sont capables de restreindre l'accumulation des protéines virales. Ces résultats suggèrent que le complexe de réplication périnucléaire formé par PlAMV est incapable de protéger de façon efficace l'accès des protéines de l'hôte au génome viral.

5.1 L'accès à l'intérieur des VRCs : prévisible ou non?

Les virus à ARN induisent la formation de complexes de réplication virale sur des membranes réorganisées de l'hôte qui servent d'échafaudages et de compartiments protecteurs pour la réplication du virus et peuvent être impliqués dans l'assemblage du virus (Tilsner et al., 2012). Les VRCs, formés par l'action de la P25 de PVX, sont des complexes périnucléaires associés au ER qui consistent en une réorganisation des filaments d'actine et des endomembranes (Tilsner et al., 2012).

L'hypothèse la plus simple pouvant expliquer l'accès à l'intérieur des VRCs serait une localisation membranaire naturelle des protéines AGOs antivirales contre PVX. De nombreuses observations documentent l'association membranaire des protéines AGO végétales et animales (Brodersen et al., 2012; Cikaluk et al., 1999; Gibbings et al., 2009; Jouannet et al., 2012; Lee et al., 2009; Li et al., 2013; Stalder et al., 2013; Wu et al., 2013). Dans un tel cas, le recrutement des membranes induit TGB1 causerait le recrutement des AGOs qui y sont associées. Parmi les protéines AGOs végétales, seules AGO1, AGO4 et AGO7 ont été testées pour leur association

aux membranes. Il a été montré qu'AGO1, mais pas AGO4, co-fractionne avec des polysomes liés aux membranes (Lanet et al., 2009), qu'elle co-localise partiellement avec le ER et co-fractionne avec les microsomes et les MBP (Brodersen et al., 2012; Li et al., 2013). Dans le cas d'AGO7, il a été démontré qu'elle s'accumule dans des aggrégats (*bodies*) associés à la membrane et que cette localisation est nécessaire pour sa fonction dans la biogenèse des siARNs (Jouannet et al., 2012). Par contre, mes travaux ont démontré que ces deux protéines, AGO1 et AGO7, sont déstabilisées par TGB1. Par une simple analyse de la séquence protéique des diverses AGOs avec les séquences d'AGO1 et AGO7, il est impossible de prédire si d'autres AGOs pourrait être associées aux membranes. Pour vérifier cette hypothèse, il serait intéressant de vérifier l'association de toutes les AGOs aux membranes. Il serait pertinent de faire les essais en conditions naturelles, i.e. chez des plantes non-infectées, et chez des plantes infectées par PVX pour vérifier si une AGO doit être préalablement liée à la membrane pour être antivirale ou si son association avec la membrane peut être induite par l'infection. Une telle relocalisation des composantes du mécanisme de l'ARN interférence induite par des virus a d'ailleurs été démontrés récemment (Barton et al., 2017). De façon alternative, nous pourrions vérifier l'activité antivirale d'AGO2 et AGO5 dans des plants dont l'expression de HMG1/MAD3 est compromise. En effet, ce gène codant pour une 3-hydroxy-3-methylglutaryl CoA réductase est une enzyme régulatrice clé qui contrôle la production des isoprénoïdes. Il a été démontré que l'association d'AGO1 aux membranes est diminuée lorsque l'on réduit l'expression de ce gène ce qui affecte la fonction d'AGO1 dans la voie des miARNs (Brodersen et al., 2012).

De façon alternative, AGO2 et AGO5 pourraient être recrutées à l'intérieur du VRC par une protéine virale. Parmi les protéines virales, seulement TGB1, TGB2 et la CP ont été montrées pour interagir avec des protéines de l'hôte. TGB2 interagit aux plasmodesmes avec une β-1,3-glucanase, une enzyme dégradant la callose (Fridborg et al., 2003). La CP interagit avec la plastocyanine qui induit la relocalisation de la CP dans les chloroplastes, avec NbCPIP2a et NbCPIP2b dans le cytoplasme et avec une NbDNAJ-like dans le cytoplasme mais aussi à la périphérie du noyau (Qiao et al., 2009; Choi et al., 2016; Cho et al., 2011). Pour sa part, TGB1 interagit avec une rémorine dans aux plasmodesmes, une caséine kinase et un facteur

d'élongation de la traduction eIF1Bβ (Raffaele et al., 2009; Modena et al., 2007; Hwang et al., 2014). La faible homologie de séquence entre ces protéines ne permet pas d'établir des prédictions. Il se pourrait néanmoins qu'un repliement semblable de ces protéines soit à l'origine de l'interaction avec les facteurs viraux. Finalement, en 2010 un groupe a démontré que TGB1 interagit avec AGO1, AGO2, AGO3 et AGO4 mais pas avec AGO5 ce qui exclut donc la possibilité que TGB1 soit responsable du recrutement des AGOs antivirales (AGO2 et AGO5) à l'intérieur des VRCs.

La composition des complexes effecteurs de l'ARN interférence, les complexes RISC, est très mal connues. Plusieurs tentatives ont été faites par divers groupes afin d'identifier ces co-facteurs chez les plantes mais ces tentatives ont été vaines. Il est néanmoins possible qu'un facteur de l'hôte, interagissant spécifiquement avec AGO2 et AGO5, soit responsable du recrutement de ces dernières à l'intérieur des VRCs.

5.2 Est-ce que TGB1 agit autrement que par la déstabilisation des AGOs

L'étude de 2010 par Chiu et al. ainsi que mes travaux démontrent que TGB1 déstabilise localement AGO1 et AGO7 (Chiu et al., 2010; Brosseau et Moffett, 2015). Néanmoins, une étude antérieure avait démontré que la P25 de PVX était un VSR qui empêchait le mouvement du signal d'extinction en dehors de la cellule initialement infectée (Voinnet et al., 2000). Ce qui démontre qu'un même VSR peut agir à différentes étapes de l'ARN interférence. Une telle multifonctionnalité a d'ailleurs été démontré pour un autre VSR, la protéine 2b de CMV. Cette protéine peut inhiber l'ARN interférence en interagissant avec certaines AGOs et en interagissant avec les siARNs habituellement incorporés dans AGO4 (Fang et al., 2016; Hamera et al., 2011). Il serait intéressant de vérifier si les autres fonctions des protéines AGOs, qui ne sont pas déstabilisées par la P25 (AGO3, AGO4, AGO6, AGO9 et AGO10) mais qui sont incapable de cibler le PVX sauvage, sont altérées en présence de la P25.

5.3 Utiliser l'ARN interférence pour conférer l'immunité

L'ARN interférence est un système largement utilisé pour la gestion des maladies en agriculture (Rosa et al., 2018). De façon traditionnelle, l'exploitation du mécanisme de l'ARN interférence pour conférer l'immunité chez les plantes est de produire des plantes transgéniques avec des séquences d'ADNc ayant de l'homologie pour les séquences codantes des virus cibles. Une telle approche a été commercialisée et utilisée avec la courge, la papaye et la pomme de terre dans les années 1990s et ce, avant même que le mécanisme de l'ARN interférence soit connu. Le désavantage d'utiliser cette méthode vient de la très grande spécificité du mécanisme de l'ARN interférence puisque cette stratégie ne pourra affecter que les agents viraux ayant une très forte homologie de séquence avec la séquence de l'ADNc introduit dans la plante.

De façon alternative, l'ARN interférence a aussi été utilisé pour réprimer l'expression de facteurs de susceptibilité chez l'hôte. Les facteurs de susceptibilité sont des protéines de l'hôte essentielles pour l'établissement d'une infection efficace par un virus. Parmi ces facteurs, on note les facteurs d'initiation de la traduction. Néanmoins, bien que ce type de résistance soit commun contre les virus de la famille des Potyviridae, elle ne permet pas une défense à large spectre.

La stratégie que nous suggérons, suite à mes travaux, est l'introduction de protéines AGOs provenant d'une autre espèce ou la manipulation de l'expression de certaines AGOs afin d'augmenter l'efficacité du mécanisme de l'ARN interférence. En effet, puisque mes travaux ont démontré que l'introduction de Col-0-AGO2 chez l'accession *Arabidopsis thaliana* C24 lui confère une immunité totale contre PVX, nous croyons que cette stratégie pourrait être utilisée pour diverses espèces d'importance agronomique, comme par exemple les Solanacées comme la tomate qui code pour une AGO2 similaire à celle de C24. Comme preuve de concept, nous avons produits des plantes transgéniques, de *Nicotiana benthamiana* et de tomate, qui expriment Col-0-AGO2 ou NbAGO2 (contrôle) sous le contrôle du promoteur endogène de NbAGO2. Les plantes ayant un niveau similaire d'expression du transgène seront inoculées avec différents virus du genre Potexvirus et Potyvirus, deux genre prévalents dans les cultures de tomate. Bien

que les résultats soient préliminaires, il semble en effet que cette stratégie permette de diminuer les symptômes induit par PepMV et PVX ainsi qu'une diminution significative de l'accumulation des protéines virales dans les feuilles non-inoculées. D'autres part, puisque la moitié des accessions analysées chez *Arabidopsis* possèdent un allèle C24-AGO2, il est possible que cet allèle confère un avantage dans la nature et qu'inversement, l'allèle Col-0-AGO2 confère un désavantage. Alors ces plantes transgéniques permettront de déterminer si la présence de l'allèle Col-0-AGO2 a un effet délétère sur le développement (vitesse de germination, biomasse, temps de floraison, production de fruits, etc).

Mes travaux ont démontré qu'AGO5 est très efficace pour compromettre l'accumulation de PVX. D'autres études ont démontré qu'AGO5 est importante pour la mise en place de la symbiose légumineuse-rhizobium chez le soja. L'expression d'AGO5 étant relativement faible, nous suggérons donc de manipuler à la hausse l'expression de ce gène, voire de le mettre sous le contrôle d'un promoteur constitutif. Cependant, cette expression devra être tissus spécifique puisque des études en cours au laboratoire indiquent que le mutant *ago5* possède une floraison hâtive, un trait intéressant en agronomie. Ces travaux démontrent qu'AGO5 interagit avec le miR156 dans le méristème apical pour réguler le développement.

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